# Engineering the rational design and optimisation of lyophilization processes for biological materials

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I, Yitzchak Grant, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:_____

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## Abstract

Lyophilization is a common method used for long term stability of pharmaceutical and biopharmaceutical products that are unstable in the liquid state for a substantial period of time. Currently, formulation and cycle development are often determined empirically. Although this approach is gradually changing as scientific publications reveal more about the nature of protein stability, nevertheless the lack of material during early stage development prevents large screening investigations to identify optimum formulations.

The use of high throughput methods coupled with factorially designed experiments enables a far more efficient and wider screening and optimisation of viable formulations for development. This thesis explores the use of micro titre plates for formulation development with emphasis on formulations for lyophilization. This is coupled with design of experiment methods to provide a powerful engineering tool for the formulation scientist.

While much has been done to model freeze drying cycles and optimize cycle parameters, current models are generic and require system specific data which can be hard to collect. By applying design of experiment principles, a system specific model was developed to allow the optimisation of cycle development to identify key parameters and produce a product that would meet critical quality attributes. Such a platform would lend itself well to quality by design and its application in lyophilization development.

# Contents

# List of Figures

# List of Tables

# Chapter 1. Introduction

Lyophilization involves the freezing, sublimation and subsequent desorption of a solvent from a product to result in a material that is dry, stable and readily soluble. In fact the term lyophilization comes from the word *lyophile* which means "*solvent loving."*(1). The principle of lyophilization or freeze drying dates back to early Incas in South America who would freeze their potato or meat on the low lying plains overnight then carry the produce up the hills in the higher altitude where the low pressure induced the sublimation of ice and resulted in a freeze dried product (2). Although no-one can claim to have invented the first freeze dryer, there are records which suggest that Nestle produced freeze dried coffee as early as 1938. The first commercial freeze-dried biological product was blood plasma for the troops during WW2 (3). With the current high value of the biopharmaceuticals market and the prediction for further growth (4) there is a high demand for well formulated biopharmaceutical products.

## 1.1 Solid State Formulation

In an industrial process the ideal product ready for shipping should be stable with a shelf life of at least two years. It should be in its dosage size ready for use by the end user. Therefore the optimum formulation would be a liquid. Furthermore, the product is generally in liquid state following final stage polishing and a liquid final formulation would require minimal further processing (each process step costs money, takes time and often incurs product losses). However for protein based biopharmaceutical products in particular, liquid

formulations tend to be unstable at ambient conditions. It is important to remember that the product can be exposed to a wide range of conditions during shipping such as low pressure and cold temperatures in an airplane or high temperatures and rough handling or agitation on a truck travelling through Africa (5).

During storage in liquid formulations proteins are mobile and free to interact with each other and with the other components of the formulation. There are many degradation pathways through which biopharmaceuticals can lose biological activity and they can be grouped into chemical instabilities and physical instabilities (6). Chemical instabilities include deamidation, oxidation or hydrolysis among others while physical instabilities include denaturation, adsorption, aggregation and precipitation. Furthermore the stability of a biopharmaceutical product can also be dependent on the final protein concentration in the dose. High concentrations can encourage aggregation, whereas at low concentrations, surface adsorption losses can become problematic. As biopharmaceuticals become more complex there exists a need to preserve them in a stable state that prevents degradation during storage. This need highlights a requirement for solid state formulations.

Biopharmaceuticals can be stored as a solid through four methods: freezing, crystallization, spray drying and lyophilization.

Freezing is often used in the laboratory for storing and preserving proteins. However the freeze thaw cycle used has to be optimized to prevent protein

degradation. The freezing itself can damage the protein and most importantly this method requires the use of a freezer for storage and transportation. As such, while it is often used as an intermediate step during bioprocessing at two different sites where it can be further purified to remove aggregates, it is not feasible for biopharmaceutical production as a final dosage form.

Precipitation is a process by which the product is "salted out" of solution in a well defined buffer and ionic strength resulting in a precipitated product. This is carried out in a stirred tank and the solution is then centrifuged to extricate the product. It is relatively cheap requiring little in the way of equipment as centrifuges and stirred tanks are often already present in production facilities. Antibiotics are an example of a product that is produced through this method. However, in relation to protein based products this method is essentially reliant on precipitation of proteins to form an insoluble precipitate. If the precipitate can be re-dissolved easily and the proteins themselves refold to their native state, then precipitation may be a viable option. In general though complex proteins do not lend themselves well to reversible precipitation and this is not a viable option.

Spray drying is a common process in both the healthcare and the food industries. A concentrated product stream is forced out of a nozzle at high pressure where a blast of hot air strips away the water layer around the droplets resulting in a fine dried powder. Although the hot air can damage heat labile proteins, the speed of the process means that this is not the primary factor that results in protein degradation (7). Rather the high level of water/air interfaces resulting from the high surface area of multiple droplets results in degradation of

the protein at high levels (6, 7). Furthermore the proteins are subject to high shear rates in the proximity of the nozzle and these are known to damage proteins (8).

An alternative to spray drying is spray freeze drying where the product stream is brought into contact with a medium such as liquid nitrogen and the frozen droplets are then dried under vacuum or a fluidized bed or a combination of both (11, 12). This process has been used to develop powders for inhalation as the porous powder particles produced re – suspend well in the respiratory tract. However, for production of single dose vials containing a dry drug product where the filling, drying and finishing all takes place in one sterile step, lyophilization can be a better option.

Lyophilization is a gentle process by which a protein is first immobilized in a solid ice structure and then dried under vacuum. If a product is stable or robust enough that one can produce a stable final stage product for the customer using any of the other methods then freeze drying should not be the formulation process of choice. Lyophilization is an expensive procedure as it involves a high capital cost investing in a vacuum chamber and an accurate freezing shelf and high running costs as it involves vacuums, low temperatures and time. Furthermore lyophilisation processes are usually run in a batch rather than continuous mode. Batch methods tend to cost more than continuous ones such as spray drying. It also means that all ones "eggs" are essentially in one basket and if the run should fail an entire production batch is lost. As such it is important to bear in mind when designing a process that lyophilization is often the last resort

approach to solid state formulation. However the delicate nature of proteins means that this last resort is often required.

### 1.2 Equipment

The lyophilizer itself is a fairly simple piece of equipment that has been unchanged in principle since its first use. A systematic diagram of a lyophilizer is shown in Figure 1.1. Some developments that have been made in the past twenty years or so are more airtight chambers, automatable systems programmed using a PC interface including data monitoring, sterilisable chambers and better vacuum pumps.

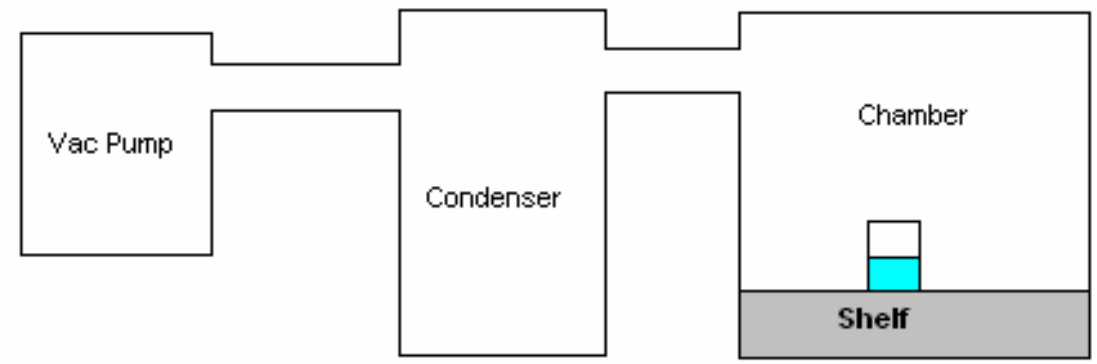


**Figure 1.1 Schematic of freeze drier with vial of product on shelf**

The product can be pre-frozen or frozen *in situ,* and thermocouples can be inserted into some vials to monitor the freezing rate. Once all the vials are frozen the vacuum pump is switched on and the pressure is lowered. Ice sublimes to vapour and is transported to the condenser which is kept at a much lower temperature than the drying chamber. The ice condenses on the walls of the condenser thereby preventing the vapour from contaminating the vacuum pump. The vial can be partially stoppered and following secondary drying, the stopper can be pressed into the vial thereby sealing the product in a dry environment.

Although the pressure within the vial is lower than atmospheric pressure, this is not a problem in fact it is preferred as it will then suck in the water from a syringe inserted for resuspension and delivery to a patient.

## 1.3 The Freeze Drying Process

When detailing the lyophilization process it is common to break it down into its component stages: freezing, primary drying and secondary drying.

### 1.3.1 Freezing Phase

The freezing stage involves freezing the product in containers until the solution changes from liquid to a solid state. The solvent, generally water, forms ice crystals with the protein and any other components squeezed into the interstitial space between the crystals. The rate at which the shelf temperature is lowered is critical in determining the crystal size. Rapid cooling rates typically result in small crystals whereas slow cooling rates result in large crystal formation. There is a trade off to consider as larger crystals can impose higher mechanical stresses on the protein structure while the high surface area of small crystals increases the size of the ice water interface and such interfaces are known to damage proteins (6, 11). From an engineering perspective, larger crystals mean larger pore sizes forming during the primary drying phase. This means the rate of flow of vapour from the ice front through the dried layer can proceed at a faster pace with some reports of an increase of up to 3.5 times the initial primary drying rate (12).

The challenge during freezing is to ensure the product freezes. As obvious as this may sound it cannot be taken for granted as ice nucleation is a random process that cannot be engineered easily. As mentioned previously, the frozen product is a mixture of crystalline ice and amorphous/glassy regions containing the protein in the interstitial spaces. In order to ensure all material is solid one has to bring the temperature down below the *glass transition temperature* otherwise known as the $T_g$'. Even at this temperature ice nucleation rather than super – cooled liquid is not a certainty and the purer the solution and the more perfect the surface of the container, the lower the chances of nucleation. While attempts have been made to force the solution to nucleate through mechanically shaking the containers in some way, no viable method exists today on an industrial scale. This means that in the case of biopharmaceuticals where the product is lyophilized in vials, ampoules or syringes, each individual container will freeze at a different time and one will have heterogeneous nucleation between the containers Furthermore, ice crystal growth continues during freezing, so containers that freeze early will have larger crystals compared to those that freeze later.

A solution to the problem of heterogeneity is through the use of annealing(12, 13). During annealing the shelf temperature is increased to about 10-20$^{o}$C above the $T_g$' after freezing and held for a period of time. This allows the crystals to grow. As this process is controllable, one can improve the quality by design of the systems by using an annealing step that results in a more defined crystal size in all vessels.

The $T_g$' of the formulation is a crucial characteristic when designing a lyophilization cycle. In fact in the past many cycles were designed purely on the knowledge of the $T_g$'. There are several analytical methods for determining the $T_g$ and these are discussed under section 1.6 Formulation characterization.

### 1.3.2 Primary Drying Stage

During this phase the pressure in the chamber is lowered to create a pressure difference between the vapour pressure directly above the ice front and the chamber. The process of sublimation of ice at low temperature and low pressure can easily be visualized using the phase diagram shown in Figure 1.2. At sub zero temperatures below 6.1 millibars the solid ice will sublime to vapour.

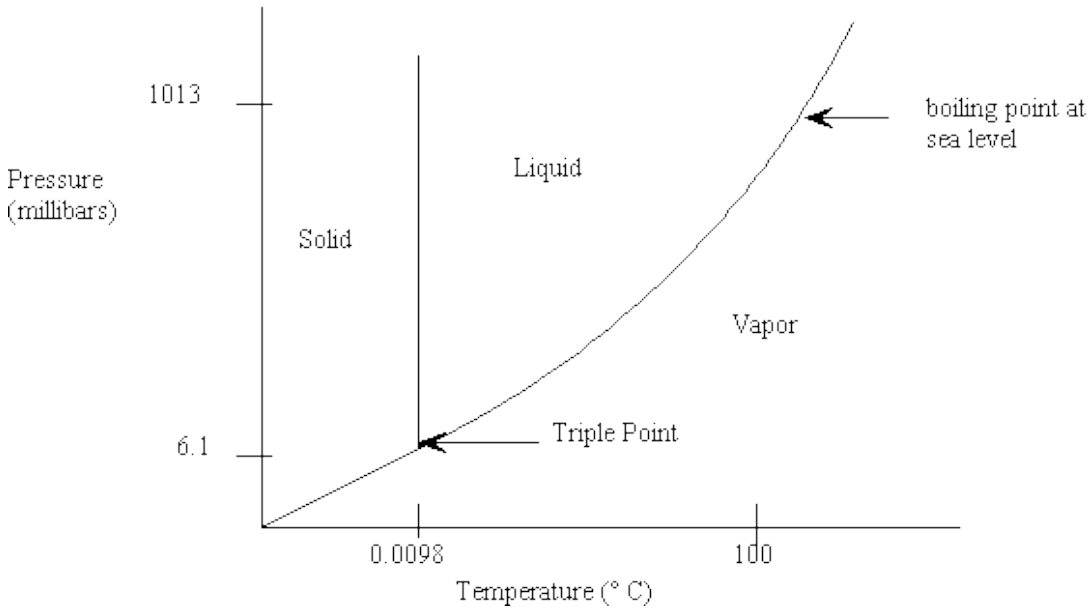


**Figure 1.2 Phase diagram for water**

The sublimation process requires energy the source of which is the heated shelf. It is essential throughout the process that the temperature of the contents of the container do not exceed the $T_g$' throughout otherwise meltback can occur. However, by carefully adjusting the shelf temperature one can provide maximum

thermal energy while maintaining a product temperature below its $T_g$'. Overall this process can be described as a heat and mass transfer process. The mass transfer is driven by the difference in pressures between the vapour pressure and the chamber pressure. However, the vapour pressure is dependant on the product temperature which will drop as the ice sublimes unless sufficient heat transfer from the shelf to the product occurs to replace the loss of energy due to enthalpy of sublimation.

The rate of vapour flow from the drying chamber to the condenser is dependant on the respective vapour pressures in each chamber. However as the condenser is generally kept at a much lower temperature than the drying chamber, the water vapour condenses back to ice on the coils of the condenser resulting in a far lower vapour pressure in the condenser than the drying chamber. Although in theory by changing the temperature in the condenser one could alter the vapour pressure in the condenser, the pressure in the condenser is likely to remain the same as the chamber pressure due to the vacuum pump to which the condenser is attached. In the event the operator raises the condenser temperature thereby raising the vapour pressure in the condenser, this would result in vapour flow into the vacuum pump. As such, the condenser temperature is not seen to be a driving factor during freeze drying.

Because the driving force behind primary drying is a pressure difference, it is logical to conclude that the greater the number of ice particles at the ice/air interface, the greater the number of particles are subject to the pressure difference and therefore the greater the rate of primary drying. From an engineering

perspective this means that one should aim to have a low aspect ratio between fill depth and surface area. It is better to have a large diameter vial filled to a depth of 4mm than a small diameter vial filled to a depth of 20mm.

It has been shown that there are three factors that resist mass transfer from container to condenser: a) the dried product layer that forms on top of the ice front; b) the container stopper if present; and c) the chamber resistance (14). The chamber resistance is generally the result of the interconnecting tube between the product chamber and the condenser. While at aggressive drying rates the chamber-condenser pathway can become an issue to the point where the vapour is travelling at mach 1 (18). Pikal et al (15) found that the dominant resistance accounting for nearly 90% of resistance was due to the dried – product layer. In light of this it is easy to understand why large ice crystals resulting in large pore sizes are important to increase the rate of primary drying.

Heat transfer generally takes place through three mechanisms: conduction, convection and radiation. Conduction is the flow of energy through molecular vibration. If we assume a slab of surface area A and thickness x as shown in Figure 1.3,

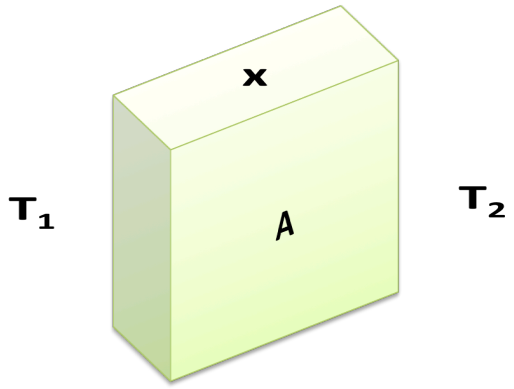


**Figure 1.3 Heat flow across a finite slab**

then the equation for heat flux via conduction through the slab can be described as follows:

$$\frac{dQ}{dt} = AkxΔT$$

**Equation 1.1 Conduction heat flow equation for a slab where Q is energy, t is time, A is surface area, k is thermal heat transfer coefficient, x is the wall thickness and ΔT is the temperature difference between one side of the slab and the other (T2-T1 where T2>T1)**

Convection relies on molecules carrying thermal energy from a source to a material, in this case from the shelf to the product via the bottom of the vial. Due to the low pressures involved in primary drying, there is a lack of molecules present to carry this out. As such the convection is not a major contributor to heat transfer during primary drying but must not be ignored. The primary method of heat transfer is through conduction, namely from the shelf through the container walls and then to the product.

Radiation does occur although when freeze drying biopharmaceuticals, conduction is by far the dominant factor. In food manufacture, heat transfer is often via radiation. Radiation occurs via infra – red and occurs between all bodies. The heat radiative heat flux from a source of thermal radiation can be described by Equation 1.2.

$$q = \varepsilon \sigma A T^4$$

**Equation 1.2 Stefan – Boltzmann Law for thermal radiation where q is heat transfer per unit time (W), ε is the emissivity (between 0 and 1), σ is the Stefan – Boltzman Constant (5.6703 x 10-8 $wm^{-2}K^{-4}$), T is the absolute temperature (K) and A is the surface area ($m^2$)**

Although in biopharmaceutical manufacture, conduction is the dominant mechanism, it is important not to underestimate the radiative effects from vial to vial and from the chamber door. Doors of laboratory and pilot scale freeze driers are often transparent and the containers closer to the door in these devices can dry faster than those towards the back. Similarly, containers on the edge of the shelf can have different drying rates from containers in the centre of the shelf (16).

Although the drying stage as a whole can be split into two distinct stages separated by their mechanisms (sublimation and diffusion), during the actual process it is not possible to determine when one stage stops and the other begins as there is a great degree of overlap between the two due to the heterogeneity formed in the sample. Secondary drying can occur during the primary drying phase. The distinction between the two is governed by the origin of the water vapour. In primary drying the vapour source is the ice crystal whereas during

secondary drying the water vapour is from the water molecules adsorbed onto the surface of the cake.

### 1.3.3 Secondary Drying Stage

As stated above, this step involves the desorption of the water molecules from the cake structure. In extreme cases this can also involve the removal of bound water from the protein super structure. This is done by raising the temperature of the product giving the ice molecules energy to sublime and diffuse through the cake structure. It is important to note that following primary drying, the $T_g$ of the product is now above zero so the shelf temperature can be raised sometimes as high as 50 $^oC$ without melting the product. While the low pressure must be maintained, there is little to gain from extremely low pressures as temperature is the determining factor for the rate of secondary drying.

Although the $T_g$ of the product is generally above zero after primary drying, the presence of up to about 25% moisture means the $T_g$ can be around room temperature. As the whole purpose of lyophilization is to produce a material that is stable for long term storage at ambient conditions, this is unacceptable as the product is likely to melt or collapse during storage at room temperature. Hence the need for a secondary drying process. Also the presence of water in the amorphous stage could allow the protein to remain sufficiently mobile to degrade by the various chemical mechanisms mentioned earlier.

## 1.4 Container systems

Several container systems exist for industrial lyophilization such as trays, syringes, vials and ampoules. In general, bulk freeze – drying trays are often used for low value products such as food or during intermediate processing steps (17). Traditionally products were most frequently lyophilized in vials which were then stoppered at the end of the run (18). Ampoules which are essentially glass sealed vials are used in some areas as the storage properties are superior (19), however as the vast majority of lyophilized biopharmaceuticals are for injection, the use of syringes is gaining growing popularity as it offers the most convenience for the end user (20-22). Furthermore, if the product is going to be placed in a syringe anyway, the vial is a wasteful method of storage. For the purposes of all investigations in this thesis, the container system is a vial unless otherwise specified.

## 1.5 Formulation

A typical formulation for a biopharmaceutical will consist of five components:

A solvent

The active pharmaceutical ingredient (API)

Bulking agent

Cryoprotectant

Lyoprotectant

It is possible and indeed quite common for one species to act as two agents such as mannitol which can act as a bulking agent and cryoprotectant. If it is

possible to freeze dry a product without the use of bulking agents, cryoprotectants and lyoprotectants, then there is no need to include them. However the majority of biopharmaceuticals require them to lyophilize. If the API and the buffer together make up less than about 2% solids the product will not form an elegant cake structure and can blow out from the top of the vial. Furthermore, the resultant cake must be well formed, readily soluble and not show evidence of shrinkage.

The typical solvent is water. This is partly because many organic solvents can damage proteins but also because the bioprocess associated with the product is generally conducted in the aqueous phase with water as the solvent. There is an advantage to using solvents with higher volatility than water as they will sublime under lower pressures during primary drying. However, this is tempered by the fact that often such solvents will have far lower melting points requiring lower shelf temperatures. Some therapeutic molecules are more easily dissolved in solvent than water and t-butyl alcohol is an example of an alternative non - aqueous solvent (23).

The water will in nearly all cases contain a buffer. Buffers are used when working with proteins to prevent unwanted pH shifts that can cause proteins to denature and/or aggregate. This is important in lyophilization as freeze concentration effects caused as the water forms into crystals and the remaining materials are forced into the amorphous regions can be quite dramatic. However, the buffer must be picked with care as it is known that some buffers (e.g. Phosphate Buffer) shift pH when frozen which can be detrimental to the process.

There is little one can do about the API as the dosage size for the end user of the product is predetermined and cannot be altered. Concentration can be an issue as at high concentrations of protein, the freeze concentration effect which can occur during the freezing stage can force a layer of protein to form a skin on the surface of the frozen product. During the sublimation stage this skin can resist mass transport until the pressure build up under the skin is high enough to blow the skin apart, perhaps out of the vial and definitely resulting in a collapsed cake structure. Should this be an issue, for example where the dosage size requires a high concentration of protein which will not lyophilize, it may be possible to lyophilize at a low concentration but high volume and then resuspend with a low volume to result in the required concentration. Otherwise there is very little the process designer can do about quantity and concentration of API in the vial.

The use of bulking agents is fairly common in situations where the solid content within the formulation is too low to form a cake. In such situations, without the use of a bulking agent, a small quantity of fine powder will form which could potentially blow out from the top of the vial. Even if it does not, the end user prefers a visually appealing cake structure which can necessitate the use of a bulking agent. A common example of a bulking agent is mannitol. An advantage of mannitol is its ability to crystallize if frozen slowly thereby providing a scaffold for the API. However if frozen too rapidly to crystallize the mannitol can expand during primary drying and break the vial (24-27).

As explained earlier, the freezing process exposes the protein ingredient to high mechanical stresses as the crystals form and the proteins are forced into the

interstitial spaces between the crystals. Furthermore pH shifts which may be occurring, the freeze concentration of salts present in the formulation or buffer are all properties that can chemically denature the protein structure. Therefore, there is a requirement for preservative materials called cryoprotectants. Examples of cryoprotectants include surfactants or polymers (5). Similarly, to protect the protein shell during lyophilization the use of lyoprotectants prevents denaturation of the protein. Various mechanisms have been proposed for the method by which lyoprotectants preserve protein structure, for example by preferentially hydrogen bonding to the protein in place of water. In any event, non – reducing disaccharides are generally the lyoprotectant of choice (5).

The process by which suitable cryo- and lyo- protectants are selected is often empirical in nature and contributes to the labelling of lyophilization as a "dark art." While the use of solid scientific understanding to develop a solution is always preferred the current knowledge regarding how these excipients interact with proteins and the mechanisms by which they protect the protein is still fairly limited. As such, a different approach is required that can allow a systematic engineering procedure to identify optimum formulations.

### 1.6 Formulation characterization

Formulation characterization includes developing an understanding of the interaction between the formulation and the API, optimizing pH, ionic strengths etc. In terms of developing the lyophilization cycle formulation characterization generally refers to thermal analysis of the formulation. An understanding of the thermal properties of a formulation is vital to provide scientific logic behind the

process design stage (28, 29). The key thermal characteristic necessary depends on whether the material crystallizes on freezing or not. In the event it crystallizes then it is necessary to obtain the eutectic point, the temperature at which the material changes from a liquid to a crystalline solid otherwise known as $T_{eut}$ whereas an amorphous material changes to a glass hence the term glass transition point written at the $T_g$'. The equipment used for determining these points are identical for both crystalline and amorphous materials with the exception of the freeze dry microscope which is only applicable to crystalline materials.

### 1.6.1 Differential Thermal Analysis (DTA), Resistance and Impedance measurement

Eutectic meters are devices that take measurements to identify $T_{eu.}$ Examples of such devices are the Lyotherm 1 and Lyotherm 2 (Biopharma Technology Ltd, Winchester, UK) which are similar pieces of equipment that identify eutectic events. Both use DTA and the Lyotherm 1 measures electrical resistance while the Lyotherm 2 measures electrical impedance. A 5 ml liquid sample is placed in a vial and frozen to -70 $^{o}$C using liquid nitrogen. For DTA the sample is then carefully heated in a heating block while the equipment measures the temperature within the sample and within a standard frozen solution (such as water) at the same time. Through plotting the differential thermal analysis one obtains a graph which shows an event at the $T_g$' or $T_{eu}$ demonstrated by the divergence in the relative temperatures as shown in Figure 1.4. Regarding electrical properties, the Lyotherm 1 measured resistance of the sample as it is heated as shown in Figure 1.5. In Figure 1.5 the $T_g$' is shown to be -33 $^{o}$C as shown by the change in the graphs curvature. Lyotherm 2 measures electrical

impedance across the sample during warming as shown in Figure 1.6. The change in curvature occurs at the point of the $T_g'$. Due to the fact that the resistance of ice is in the mega-Ohm region, impedance gives a more reliable result. The research work behind both the Lyotherm 1 and 2 has been conducted by Louis Rey and is well documented (30).

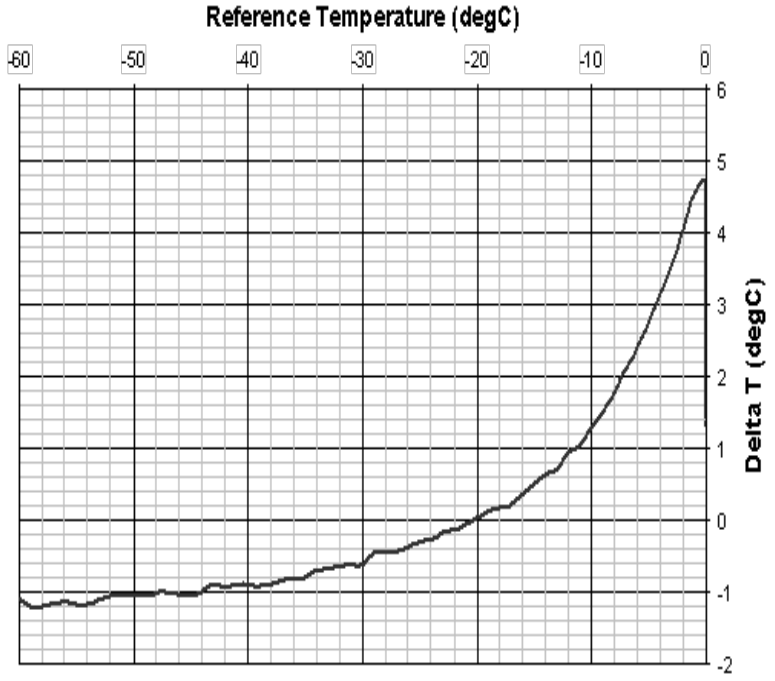


**Figure 1.4 Differential thermal analysis chart (36)**

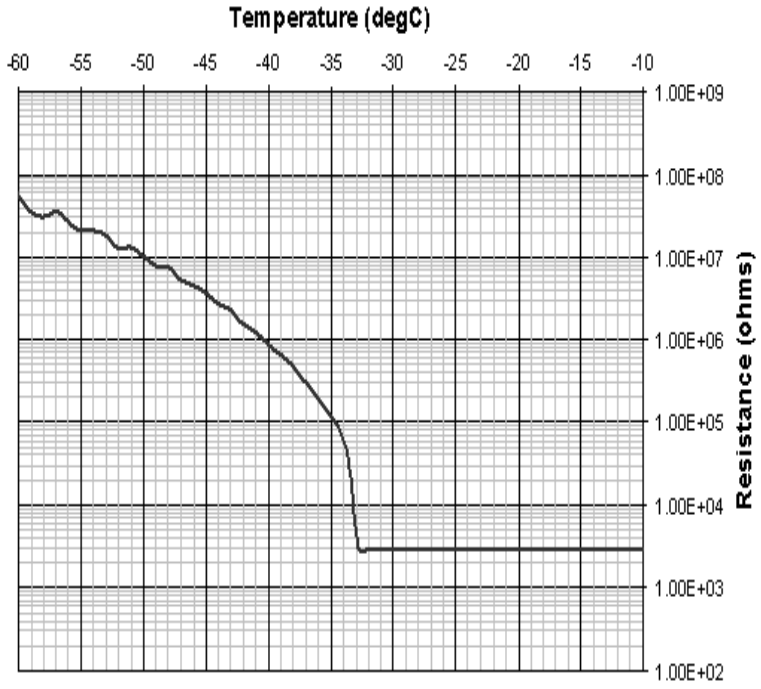


**Figure 1.5 Identification of $T_g$' using electrical resistance measurement (36)**

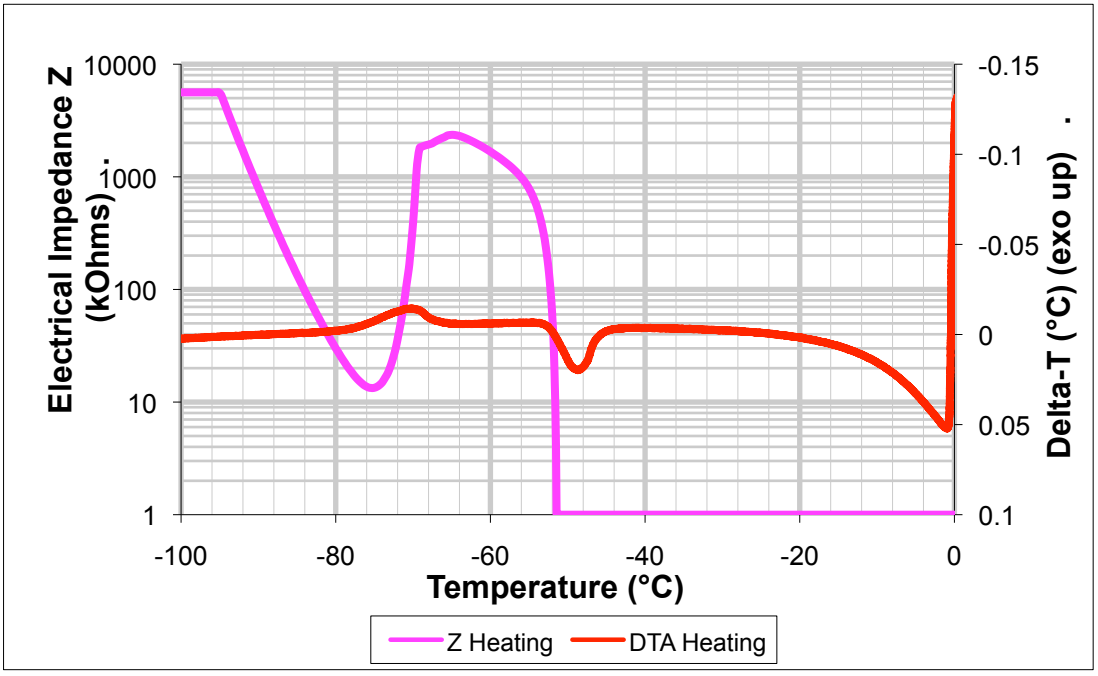


**Figure 1.6 Example of an impedance and differential thermal analysis warming profile for an aqueous solution of 5% (w/v) calcium chloride. Below -75 $^{o}$C the amorphous $CaCl_2$ (formed due to quench cooling in $LN_2$) is becoming increasingly mobile as the temp increases, but crystallises between**

**-75 and -65 $^{o}$C which shows up as an exotherm in the DTA. The eutectic temperature of $CaCl_2$ crystals is around -53 $^{o}$C where you seen the impedance drop to minimal (full mobility) and the DTA shows the melting as an endotherm. (31)**

### 1.6.2 Freeze dry microscopy

As the name suggests, this technique involves observing a sample as it freeze dries under a microscope. A drop (2–5 μL) of the sample is placed under a microscope slide and the slide is then placed on a heating/cooling shelf. Liquid nitrogen is then cycled through to freeze the sample in situ under controlled conditions. The microscope uses polarized light to allow the user to clearly identify when the material has frozen as shown in Figure 1.7.

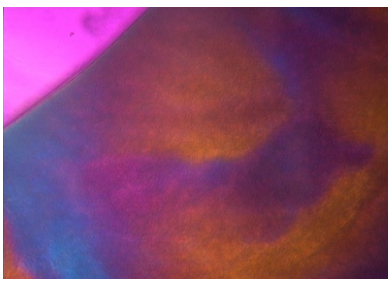

**Figure 1.7 Sample of plasma and calcium chloride frozen under a freeze dry microscope.**

Once frozen, a vacuum is applied and the temperature of the sample is raised at a controlled rate. The controller can observe the sample lyophilizing on the microscope as shown in Figure 1.8. The procession of the dried front is clearly visible as the coloured region changes to grey due to the sublimation of the ice crystals which produce dazzling colours under plane polarised light.

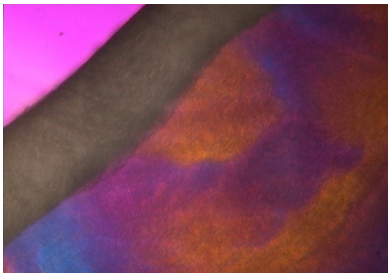

**Figure 1.8 Sample of plasma and calcium chloride lyophilizing under vacuum. The grey region is caused by the absence of water resulting from lyophilization**

The sample is continually heated under vacuum until the operator observes a situation similar to that seen in Figure 1.9. In this case, the sample temperature has exceeded the collapse temperature ($T_c$) of the sample and meltback occurs at the dry front. This allows the operator to determine the maximum product temperature acceptable during primary drying.

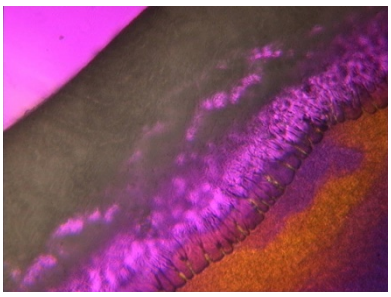

**Figure 1.9 Image showing the collapse temperature of the sample**

### 1.6.3 Differential scanning calorimetry

This method involves the measurement of heat flow through a sample as it is heated. The science behind DSC and Modulated DSC has been well documented previously (32-36). As with the Lyotherm the operator is looking for an 'event' on a trace signifying either a glass transition temperature or a eutectic event. Figure 1.10 is an example of the trace one can expect from both amorphous and crystalline materials. The crystalline material gives a clear $T_{eu}$ at -21.18 $^{o}$C

whereas amorphous materials result in a less clear 'event' labelled in Figure 1.10 at -29.29 $^{o}$C.

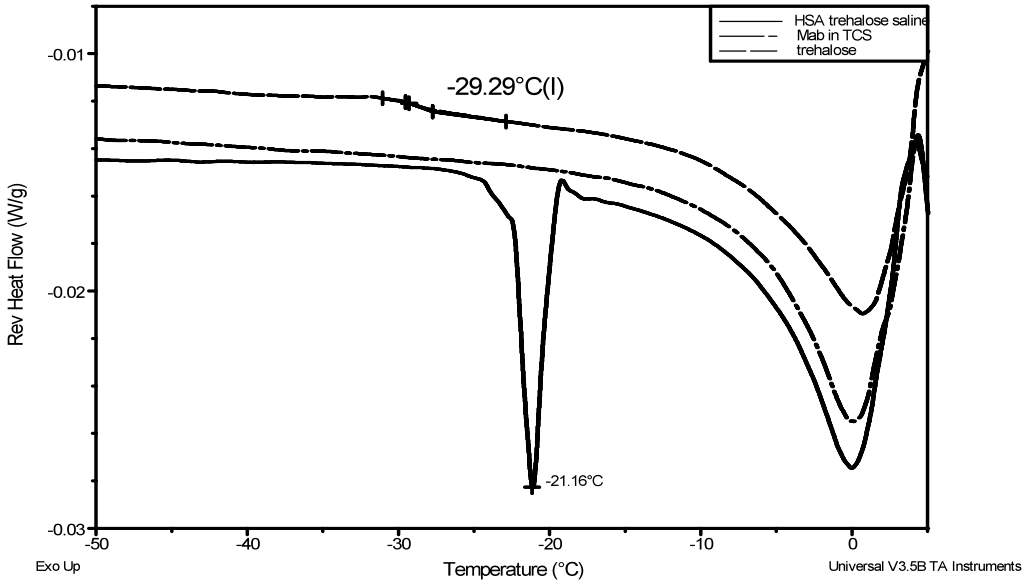


**Figure 1.10 Modulated DSC thermogram of three materials**

The primary use of all these methods is to determine the maximum allowable product temperature that is acceptable during primary drying before meltback occurs. In practice this defines the maximum shelf temperature for reasons that will be discussed in the following section. These methods can also be used to gather further detail about ones system such as how to anneal for larger crystals, or potentially to mimic the freeze drying process at low scale through the electron microscope (although research is necessary to understand how to scale up from 10 µl to 1 ml). DSC/MDSC is also used for product characterisation to understand the effect of storage temperatures.

## 1.7 Modelling the Lyophilization Cycle

The lyophilization process is a coupled heat and mass transfer process. In order for water molecules to sublime they must have the energy to do so and there must be a driving force behind the movement. The rate limiting step behind the driving force in primary drying is different from that in secondary drying (37). Therefore the implication on process design modelling means that each stage must be treated differently. For example it is known the driving force behind primary drying is the difference in vapour pressure between the drying chamber and the vapour pressure of ice at the shelf temperature. Therefore, the chamber pressure can alter the rate of sublimation. However, in secondary drying Pikal (37) found that below 0.2 mmHg the drying rate was independent of the chamber pressure and depended more on temperature. This proves that there are two different mechanisms occurring during freeze drying (38) and each one dominates at a different stage.

Modelling a coupled heat and mass transfer process with two different dominant mechanisms is complex. There have been several models proposed (17, 39-43), but all of them rely on a generic system where assumptions are made about the product such as cake porosity, resistance to vapour flow etc. Furthermore, the models involve the use of complex mathematics which the average formulation scientist may not be acquainted with. The SMART freeze dryer™ uses manometric temperature measurement coupled with a steady state mathematical model (44) to give the user a product cycle. However, the modelling is conducted automatically by dedicated software and requires a capital investment for the dryer and associated software.

Early models were based on the Uniformly Retreating Ice Front Model (45) and were refined by later scientists but such models ignored vial geometry and temperature gradients within the vials rather assuming a steady state (46). In such models it is assumed that all energy transferred into the system is used to change the state of water from ice to vapour. This ensures the product temperature does not change. The models show that in the event that the sublimation cannot occur fast enough, the product temperature will increase beyond its Tg' and meltback of the product will occur. Work carried out with pure ice demonstrated the unsteady nature of the ice front and lack of linearity in the shape of the ice front during sublimation (47,48).

There exists a need for a system specific model that would allow the user to account for variations and demonstrate quality by design as shall be explained in more detail later.

### 1.8 Process Analytical Technology (PAT) and freeze drying

The Food and Drug Administration (FDA) promote the use of PAT in all areas of bioprocessing defined as a "system for designing, analyzing and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in – process materials and processes, with the goal of ensuring final product quality (49)." The ability to understand what is happening during a process helps validate the process and ensures consistency of product and process. Such monitoring technology must not interfere with the contents of the process stream in anyway, as such it must be non – invasive, non –

destructive and non – disruptive. Such technology allows the process engineer to demonstrate control over their process. The vast majority of freeze driers come with some PAT as standard and allow the user to monitor the shelf temperature, condenser temperature and pressure within the system. However PAT in relation to the product itself is an area that requires further work.

The simplest form of PAT commonly used in lyophilization are thermocouples placed within the vial. These probes are either wired or wireless and measure the product temperature during freeze drying. Once drying is complete the product temperature is observed rising above the shelf temperature due to the fact that the energy the vial is receiving from the shelf via conduction, or the energy by radiation is not longer being used for sublimation. This causes the product temperature to rise as shown in Figure 1.11 where the product temperature can be seen rising above the shelf temperature from 20hrs. Temperature probes are relatively cheap, easy to use and most operators are familiar with the concept of thermometers and can understand the output. They allow timely measurements of critical parameters and give an indication of what is happening during the process.

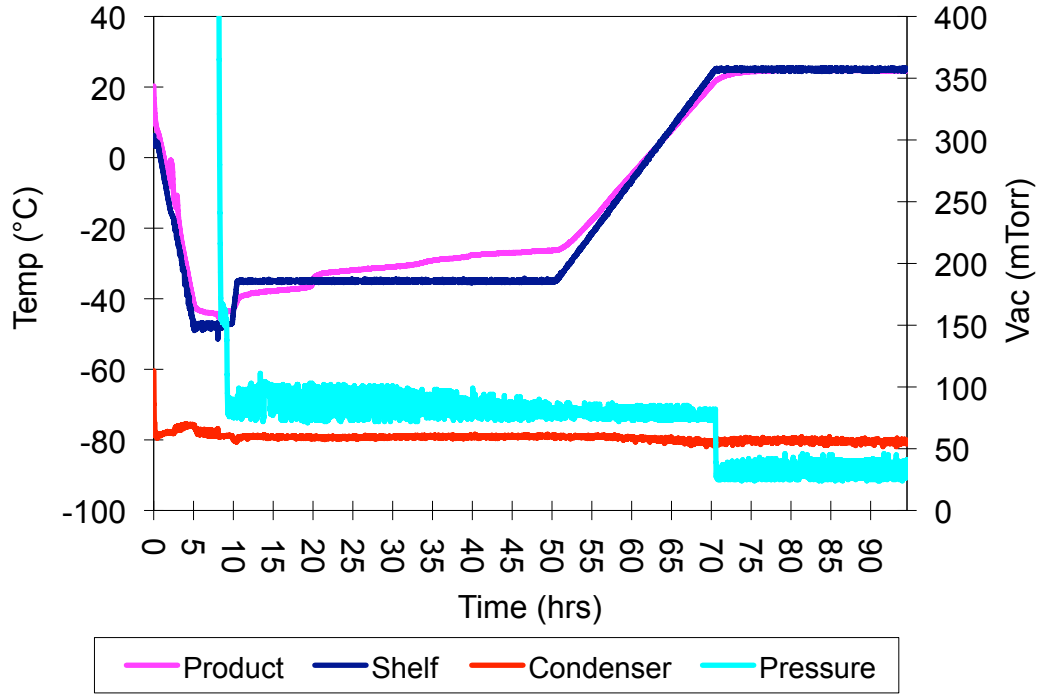


**Figure 1.11 Typical lyophilization profile (This example is from a flu antigen trial)**

Although the use of thermocouples does allow the user to see the endpoint out of hundreds of vials only 3-10 of them will have a thermocouple in them. Furthermore, the very presence of the thermocouple disrupts the ice crystal structure so the vials with thermocouples in them behave differently from those without. Finally, the vials with probes in are thrown away after freeze drying – the only vials about which the controller knows for certain what has happened are not even used! As such, although they are simple and cheap, they do not fulfil all the requirements of PAT.

The microbalance technique has been documented by Pikal et al (50) where the proposed method involves the use of a small device, which periodically picked up and weighed a vial then put it back down on the shelf. Through the use

of this device Pikal et al determined the primary resistances to mass transfer during primary drying amongst other important results. As a PAT the mass balance is non – invasive and non – destructive and the vial can be used at the end of the process. However although the vial is only lifted off the shelf for a short time, the accumulated effect of multiple lifts from the shelf may have an impact on the heat transfer to the product. Neighbouring effects from nearby vials are non existent as the vial to be lifted is isolated from any neighbours. Also this only measures one vial and there is documented evidence that shows vial – to – vial variability occurs. Therefore until someone develops a method of weighing all the vials directly in the dryer, this is not an optimum PAT.

An early novel form of PAT in lyophilization was the windmill device (51). This involved the use of an aluminium impeller with a needlepoint axle and sapphire bearings to reduce friction as much as possible. The pressure difference caused by the flow of water vapour would result in a spinning windmill. When the operator saw the windmill stop spinning he would know the end of the primary drying phase had been reached. This meant the operator needed to regularly check the windmill visually. Experiments showed that the windmills continued to spin after the product temperature reached the shelf temperature suggesting that they were still spinning after the primary drying phase had ended. This could have been due to the frictionless bearings. Either way it was not considered a robust enough method to pass regulatory approval.

It has been shown that it is possible to monitor the rate of sublimation within a vial using Fourier Transform Infrared Spectroscopy. This method

involves the use of a non – invasive probe which can monitor the quantity of ice remaining in the vial. However, due to the size and type of probe there are issues involving sterility and ease of use. While these issues are not insurmountable the end result would still be a technology that only measures one vial in 500 which would not give the user sufficient information to demonstrate control over the system.

The pressure rise test has been shown to allow the user to determine the end point for lyophilization (5). In this method, the valve between the condenser and the drying chamber is closed. The vacuum pump is behind the condenser so the valve shuts off the connection between the pump and the drying chamber. The pressure within the chamber rises due partially to unavoidable leaks but primarily due to the vapour subliming off the vials. This continues until the pressure in the chamber reaches the vapour pressure of the ice front in the vial. By correlating this with thermodynamic data tables one can work out the temperature of the ice front. Furthermore, should the pressure not change at a quicker rate than the leak rate of the chamber one can infer that the drying has finished. A major advantage of this is the lack of requirement for any additional equipment assuming one has a valve between the chamber and condenser. Another method includes manometric measurement comparisons between a Pirani and Capacitance manometer. The Pirani Gauge is known to be slightly inaccurate due to the presence of water vapour whereas this does not affect the capacitance manometer. Therefore when both are present in line with the freeze dryer system, if they both read the same measurement one can infer there is no water vapour and the drying process is over. Through combining these two methods, the Smart Freeze Dryer was

developed (52) which optimizes the cycle based on the data received through a combination of the two above methods. While this is undoubtedly a great achievement in the lyophilization field, it is a batch – based system in the sense that it only gives information about the overall batch rather than individual vials which still leaves room for further development.

Another batch method of PAT worth mentioning involves the insertion of an infra – red spectrometer or a mass spectrometer in – line between the chamber and condenser. By analyzing the process stream between the two one can work out when the endpoint has been reached. However this involves retro – fitting an existing system and validating it in terms of both sterility and leak rates. Furthermore it is again only a batch analytical technology.

This discussion not only highlights the requirement for PAT in lyophilization but also highlights the difficulties involved with lyophilization. The issue is that unlike most if not all other bioprocesses where there is one reactor, one chromatography column, one membrane, one system, in lyophilization each vial is an individual system/reactor on its own making lyophilization effectively hundreds or thousands of small unit operations each requiring their own PAT.

### 1.9 Quality by Design (QbD)

Whereas in the past, process validation protocols demanded process parameters were kept constant to ensure consistent product development, regulatory bodies came to accept that it is impossible to ensure no variation occurs in the lifetime of a bioprocess. Suppliers of raw materials can change, accidents can happen and further scientific knowledge can impact on design. The emphasis

shifted onto ensuring a consistent product which does not automatically imply a consistent process. The FDA coined the term design space defined as the "multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality (53)." This meant that the process designer must have a demonstrated knowledge of the sensitivity of the process to variations in the process parameters. Having identified the region of operability within the design space, any variation within the region is not considered a change and would not then require the initiation of a regulatory post approval change.

This means that when designing lyophilization processes one can account for variabilities in the process so long as previously documented evidence exists to demonstrate safety and consistency in product quality. The use of designed experiments to map the design space can be an invaluable tool as explained in section 1.10.

### 1.10 Ultra scale down and design of experiment

The use of factorial design of experiment (DoE) software is becoming increasingly popular in industry for screening and optimising processes, particularly in fermentation. Such methods have been shown to be advantageous in rapid screening experiments compared to traditional systematic one-factor-at-a-time (OFAT) approaches (54). Ultra scale-down (USD) studies have been used for many industrial processes such as fermentation (55), centrifugation (56), microfiltration (57), chromatography (58), and protein refolding (59), and also for studying fundamentals such as mixing (60,61), and the thermostability of proteins

in response to environmental conditions and additives (62) as well as formulation (63-65). The large datasets that can be rapidly accumulated with these methods lend themselves well to DoE methodologies. For example, ultra scale-down of fermentation has also been recently carried out in conjunction with statistical experimental design (54) to achieve optimisation of soluble protein expression in *E. coli*.

Factorially designed experiments allow the user to cover a far wider area of the design space that traditional one factor at a time (OFAT) approaches. For example Figure 1.12 demonstrates a traditional approach to formulation design. An optimum is found for trehalose while keeping other excipients constant. It is then held at the optimum and the next excipient under investigation is varied and so on. As Figure 1.12 shows, vast areas of the design space are left unchartered. Furthermore, if there is an interaction, ie if the whole is greater than the sum of the parts, one will never identify such an interaction by varying one factor at a time. However by varying all the factors at the same time as seen in Figure 1.13 one gains a much better understanding of the design space thus increasing the efficiency of process development.

DoE also lends itself well to validation. It allows the user to understand the entire design space and conduct sensitivity analysis to understand the impact of variations. However DoE can result in multiple experiments. This is where ultra scale down is useful. As has been mentioned before elsewhere, during early stage development the quantity of material is often limited. The use of ultra scale down methods allows more data to be gathered from less material. By combining this

with DoE one can really streamline process development. Furthermore, ultra scale-down methods involve the use of microtitre plates, a standard format for which robotic systems already exist. In other words, it lends itself well to automation which is a major advantage.

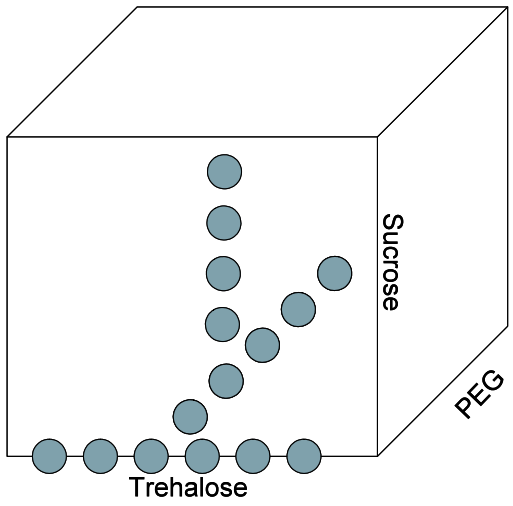


**Figure 1.12 OFAT approach to formulation development**

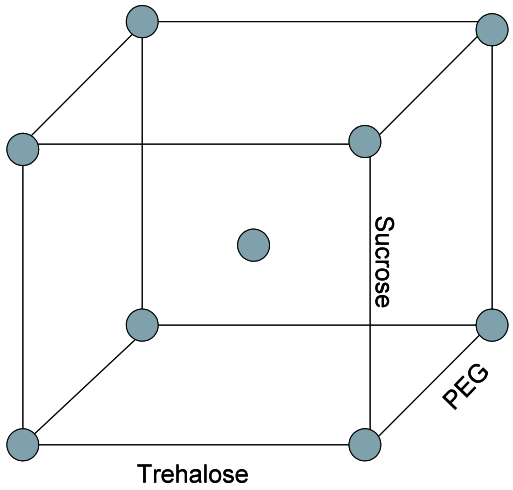


**Figure 1.13 DoE approach to formulation development**

### 1.11 Aims of this thesis

Currently the methodology used to identify optimum formulations and drying cycles is highly empirical. Formulations are often chosen on the basis of experience (it has worked with something else) sketchy scientific knowledge (this

formulation works in liquid form) or even at random. The current literature does not contain any systematic approaches to formulation development. Current research methods aim to determine relationships between classes of biopharmaceuticals and excipients. The aim of these methods is to utilize such relationships to predict ideal formulations. However with the increasing complexity of antibodies manufactured for therapeutics, the ability to classify antibodies is limited. Genetic modification of antibodies make classification even harder and with the introduction of peptides an intelligent targeted approach to formulation development would be ideal. It is unlikely that such an approach will exist within the near future.

Drying cycles are frequently over-engineered resulting in unnecessary lengthy cycles. Although the steady state model allows prediction of a drying cycle and associated parameters, it involves estimating critical parameters such as resistance to mass flow. More importantly, the intelligent freeze dryer which utilizes steady state models and the pressure rise test to determine cycle parameters, does not show where the edges of failure are. Indeed, the recommended cycle could even be on the edge of failure. In terms of QbD, this is not ideal.

This thesis aims to develop a systematic step - by - step approach which the engineer can use to develop and optimise a formulation for lyophilization. Such a platform technology would allow the user to optimise formulation development easily and gain sufficient understanding to allow process validation as well as an optimum formulation. Furthermore, the use of DoE will be used to

develop an empirical specific process model to allow the user to optimise the lyophilization cycle. The resultant model will provide a detailed map of the design space showing the edges of failure. Such a method allows for a quality by design approach to cycle development which, until further steps are made in PAT, are vital in lyophilization development.

### 1.12 Objectives of this thesis

- To identify scale up factors between microplates and vials
    - Freeze dry water in different scale containers
    - Compare results to identify scaling parameters
- Develop a high throughput platform technology for formulation development
    - Working in microwells, utilize DoE to screen for and optimize an ideal formulation
    - Validate the resultant formulation in full scale containers
- Validate that the formulation development platform developed works for more complex products
- Utilize DoE to develop a generic model for cycle development

# Chapter 2. Investigation into the Key Parameters That Effect Sublimation Rate at Different Scales

## 2.1 Introduction

Ultra scale down is a technique whereby industrial scale bioprocesses are mimicked on such a scale that microlitres of material are used. Much work has been carried out to date using such methods to predict process conditions on scale up from microscale to pilot scale to industrial scale. Work done to date has included studying the impact of shear on microbial cells (66), optimisation of cell recovery processes such as filtration and centrifugation (56-57, 67), expanded bed adsorption chromatography (68) and protein stability studies (62). By minimizing the volume of material used such methods increase the efficiency of research and development investigations in terms of data gained per gram of material used. To date, little work has been carried out using ultra scale down to optimise freeze drying. Some work has been carried out in PCR well plates (69) however the shape of PCR wells does not lend itself well to effective heat transfer during lyophilization, or correctly mimic lyophilization vial geometry. Currently work carried out in the lab and pilot scales to optimise freeze-dried formulations and processes is performed in the same sized vial as those used in an industrial scale lyophilization run, from which a larger process is achieved by scaling out rather than scaling up. This process of scaling out is not without its own factors that are scale dependant. Each vial is essentially a semi-independent mini-lyophilization process and as such, one can expect variations to occur between vials. For example, the heat transfer into the sample is known to vary depending on the positioning of the vial on the freeze-dryer shelf. The heat transferred by radiation

is known to create edge effects whereby the outer vials are heated more quickly resulting in faster rates of sublimation (14, 16, 68). Furthermore, the initial stage of lyophilization is a non-uniform and partially random event where different vials, especially those containing different formulations, can freeze at different times and temperatures resulting in different sized ice crystals (70). Having multiple reactor vessels make Process Analytical Technology (PAT) for freeze-drying extremely challenging (71), although solutions such as the pressure rise test which measures the vapour pressure of water present in the chamber at any one given moment in the sublimation process (72) have shown themselves worthy of use in commercial processes.

The determination of viable formulations and processes for freeze drying is still done largely empirically. New biopharmaceutical products are typically compared to similar existing lyophilized proteins for which a good formulation is already known, and the new formulation obtained by an iterative adaptation of the previous one. While a great deal of fundamental work has been done to increase our understanding of the mechanism by which different excipients act as cryo- and lyo-protectants (31, 73-76), the variety and novelty of new protein based products have resulted in a critical need for a more systematic screening process for the identification of viable formulations that preserve the product during lyophilization. Such a process would have to use minimal quantities of material as the research would be carried out on 99.99% pure product during early process development when any product available would be costly and available in small quantities, and also needed for the clinical trials. Conducting such research at the ultra scaledown level in microtitre plates presents a clear advantage provided a

suitable mimic for larger vials can be found. As well as the advantage of using minimal material, microtitre plates have the added advantage that they lend themselves well to automation as robotic equipment already exists that uses the microtitre format.

Before the data from research carried out in microtitre plates can be relied upon, the factors that are affected through lyophilization at such a small scale must be identified and characterized to demonstrate that they behave similarly upon scale – up to the vial. The factors that influence the rate of freeze drying within vials have been identified and well characterized in terms of heat transfer into the vial, sublimation at the ice front, mass transfer out of the vial, and resistance to vapour flow (14). Heat transfer from the shelf into the vial is partly influenced by the heat transfer coefficient of the material the vial is made from. As vials are made from glass and the microtitre plates used made from plastic and as each vessel had difference wall thicknesses, this had to be taken into account. Resistance to vapour flow is primarily dependant on the porous cake formed by the freeze dried product (14). During this investigation the process was simplified as the product investigated was pure water. This removed the resistance to vapour flow factor and the secondary drying stage completely as no residual cake would be left after the water had sublimed.

In any process it is important to characterizing the reactor vessel and lyophilization is no different (14, 77). Furthermore when scaling a process down one has to characterize the scale down system and understand how it relates to the full scale reactor vessel. Otherwise data gathered at the scale down level cannot be

applied to full scale and does not achieve anything. This investigation looks at the key factors that affect the sublimation rate of ice under vacuum in different vessels. The results give an insight into the parameters that must be controlled during scaling the process up or down during primary drying. Using the results from this investigation, valid predictions will be made based on comparing scale-down experiments to the larger scale systems.

## 2.2 Materials and Methods

Three vessel types were used during this investigation: 5 ml glass vials, 3 ml glass ampoules and wells within 96-well flat bottomed polystyrene Sarstedt microtitre plates. The physical dimensions of the vessels and the fill volumes used are shown in Table 2.1. All three vessels had the same cylindrical geometry although the diameter to height ratios differed. The fill depth was kept constant within all vessels to ensure that heat transfer from the shelf to the material was the same across all vessel types.

| | Vial | Ampoule | Microwell |
|---|---|---|---|
| Diameter (mm) | 21.15 | 13.65 | 6.38 |
| Cross Sectional Area (square mm) | 351.18 | 146.34 | 32 |
| Fill Volume (ml) | 1097.43 | 0.46 | 0.1 |
| Liquid Height (mm) | 3.13 | 3.13 | 3.13 |
| Wall Thickness (mm) | 1.1 | 0.6 | |
| Construction Material | Glass | Glass | Polystyrene |

**Table 2.1 Physical dimensions and fill volumes for vials, ampoules and wells**

Five vessels of each type were filled with the corresponding volume of Reverse Osmosis (RO) water. The vials and ampoules were then weighed and all three sets of vessels were placed on the shelf of an Edwards Modulyo Freeze drier

(Edwards BOC, Crawley UK) frozen *in situ* to -20 $^{o}$C and held for 1.5 hours. A vacuum of approximately 85-100 µbar was then applied for 20, 40, 60, 80 or 100 minutes to all vessels. The vacuum was released, the remaining ice was then allowed to melt at room temperature and the remaining volume of liquid was determined either by weighing again in the case of the vials and ampoules or by volumetric determination using a gas tight 250µL gastight Hamilton Syringe.

### 2.3 Results and Discussion

The results of the sublimation experiments for all vessel types can be seen in Table 2.2, however they can be compared more clearly in Figure 2.1.

| | Mass Lost (mg) | | |
|---|---|---|---|
| Time (mins) | Microwells | Ampoules | Vials |
| 0 | 0 | 0 | 0 |
| 20 | 35.4 | 68 | 138 |
| 40 | 56.4 | 136 | 256 |
| 60 | 69 | 176 | 296 |
| 80 | 98 | 258 | 474 |
| 100 | 116 | 344 | 574 |

**Table 2.2 Results of sublimation experiments**

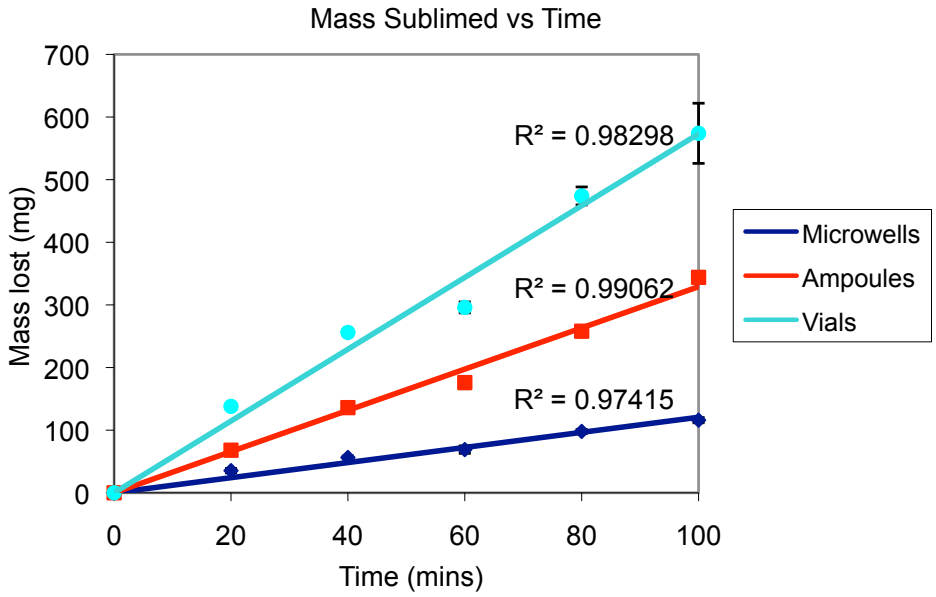


**Figure 2.1 Average sublimation of ice in 5 microwells, 5 ampoules and 5 vials**

At all scales the mass of water lost by sublimation remains proportional to the time for which sublimation was allow to proceed. The vials showed the greatest rate of sublimation, followed by the ampoules and then the 96-well microplates. This trend was expected as the vials had the widest base diameter thereby providing the greatest surface area in contact with the freeze-drier shelf. This surface area has already been identified as a key factor in influencing the rate of sublimation (14, 70) and the relationship between surface area and sublimation rate is given by Equation 2.1. The gradient of the lines in give the water sublimation rates for each vessel in mg/min which Equation 2.1 shows is affected by the surface area (A). However the other parameters in Equation 2.1, i.e. vapour pressure, chamber pressure and the resistance to vapour flow, were the same for all vessels used.

Figure 2.2 shows a plot of the sublimation rates calculated using the results from Figure 2.1 against the surface areas of each vessel. As expected from Equation 2.1 there is a linear relationship between the surface area and the sublimation rate where the sublimation rate increased with surface area.

$$\frac{dm}{dt} = \frac{A(P_0 - P_c)}{R}$$

**Equation 2.1 Sublimation rate during primary drying where dm/dt is the rate of mass transfer, A is the cross sectional area of the vessel, $P_0$ is the equilibrium vapour pressure of ice at the temperature of the frozen material, $P_c$ is the chamber pressure and R is the resistance to vapour flow by dried product**

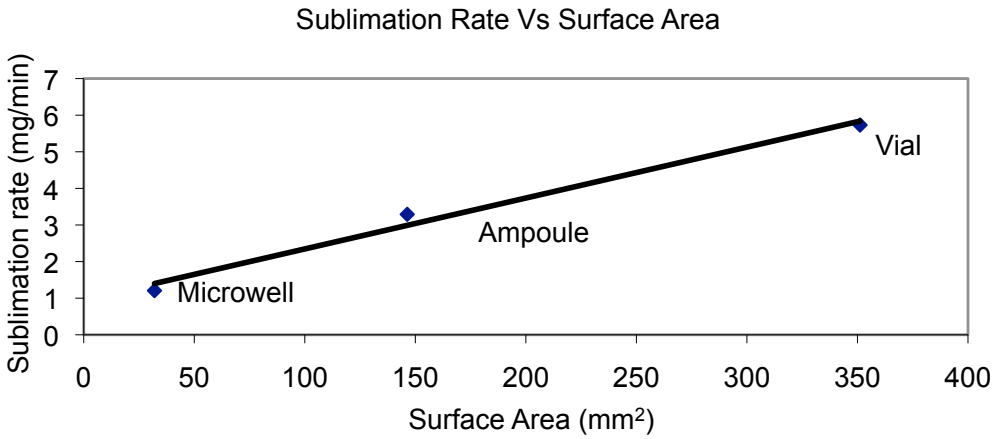


**Figure 2.2 Linear relationship between sublimation rate during freeze-drying and the surface area of the vessel in contact with the freeze-drier shelf**

That the sublimation rate increased with an increase in the surface area is not surprising. A higher surface area means that there are more ice molecules at the air/solid interface that are able to sublime at any one moment. It would therefore be expected that the specific rate of sublimation, i.e. the rate of sublimation per unit area, should remain constant regardless of the vessel. However this is not the case as shown in Table 2.3, and also because although the relationship in Figure 2.2 is linear, the plot does not pass through zero. The microwells have a far higher specific rate of sublimation than for ampoules, which in turn is slightly greater than the vials which have the greater surface area.

| | Sublimation Rate Per Unit Area |
|---|---|
| | mg/min/mm2 |
| Microwell | 0.038 |
| Ampoule | 0.022 |
| Vial | 0.016 |

**Table 2.3 Sublimation rate per unit area for three vessels**

One could argue that the differences in specific sublimation rate are due to variations in the thermal transfer coefficients of the materials that the vessels are

made from. However, the thermal transfer coefficient of glass is actually greater than the polystyrene of the microplates, and the thickness of all three vessels are comparable. Furthermore, the ampoules and vials are both made from the same type of glass, and so one would expect them to have similar specific sublimation rates. Visual observation of the recession of the ice front during sublimation reveals that, contrary to popular models used to predict sublimation rates (14, 50, 52, 77-78), the ice front does not recede uniformly but shrinks away from the walls of the vessel as well as from the surface of the ice. This implies that sublimation at the edges of the air/solid surface of the sample is more rapid than at the centre of that surface. This could be due to a combination of two factors. Firstly, the walls of the vessel are made from hydrophobic materials. It is reasonable to assume that the bonds between the ice molecule and the wall at the ice-wall interface are weaker than the ice-ice interactions in the centre of the vessel. Therefore less energy is required to break them and allow the ice molecule to sublime. This leads to the second factor which is that ice is an excellent insulator, which suggests that there must be a thermal radial gradient across the ice making the ice in the centre colder and less likely to sublime.

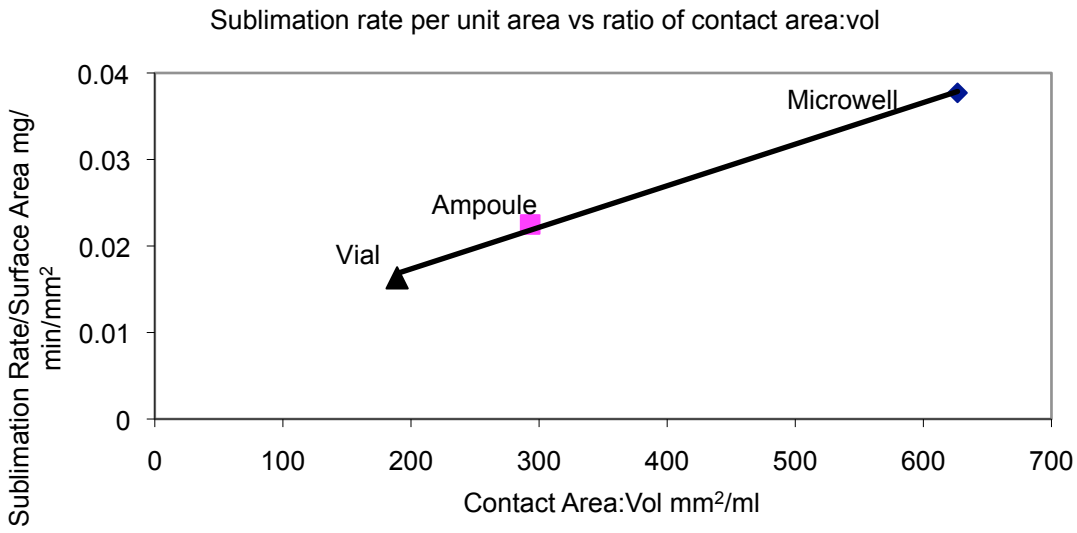


**Figure 2.3 Relationship between contact area to volume ratio and specific sublimation rate**

Looking at the ice – wall interface factor more closely the influence can be observed by relating the surface area of this interface with the total volume of the fluid. All the vessels were placed under the same conditions and lyophilized at the same time and all the vessels had the same fill depth of ice. As expected the vials which had the largest diameters sublimed the fastest. However, if we look at the sublimation rate per unit area one would expect them all scales to be the same, whereas Table 2.3 shows microwells sublime faster. By combining this fact with the ice – wall interface theory one can understand that the microwells are smaller units and as such have a larger ice – wall interface surface area than any other vessel. Figure 2.3 demonstrates this relationship well as it is a plot of the ratio of ice – wall interface to the volume of fluid within against the specific sublimation rate. Essentially the ice – wall interface to volume ratio is a measure of surface area. Surface area is known to be an important factor in any reaction – ground powder reacts quicker than large particles. So too, microwells "react faster" or

sublime quicker than vials. This is important because it implies that the presence of a probe or anything that increases the ice – wall interface area will speed up the sublimation.

Furthermore, as the ice immediately next to the wall of the vessel sublimes, the remaining ice comes into direct contact with the air. Although one may picture the water vapour rising from a flat ice front a more realistic picture is a conical ice front where the vapour flow travels in multiple directions as shown in Figure 2.4.

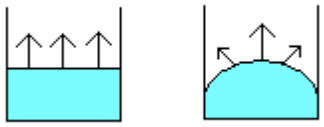

**Figure 2.4 Direction of vapour flow with a flat ice front and a conical ice front**

## 2.4 Conclusion

This investigation aimed to understand the parameters that need to be understood when using microwells as a scale down mimic of a vial based system and assess the validity of any data gained. Through this investigation it has become clear that it is possible to freeze dry materials in microwells and that the results could potentially be used to predict large scale operations. Although the rate of sublimation is different in microwells from vials, this can be explained and measured by determining the size of the ice-wall interface in relation to the total volume of ice. Once that is determined the relationship between that and the specific sublimation rate is a linear one and predictions can therefore be made

from one vessel to the other. However, this has been difficult to prove for a product containing solids due to the limitations of Karl-Fischer titration in relation to microtitre plate freeze drying. The titration required the mass of solid present in the well which is in the order of micrograms. However the plate weighs approximately 10 grams and there was no balance available that could measure 10 grams to the precision of a microgram.

The sublimation of ice requires energy which is supplied primarily through conduction (14), but convection and radiation are important factors too. Conduction occurs from the shelf through the bottom of the vessel into the ice. According to the second law of thermodynamics, wherever there is a temperature difference, thermal energy will spontaneously flow from the hotter body to the cooler body. Therefore, if the vessel itself is at a higher temperature than the ice within, thermal energy must transfer from the vessel wall to the ice. A higher temperature of ice means a higher vapour pressure which in turn means the pressure difference between the vapour pressure of that ice particle and the chamber pressure is higher thereby promoting a faster sublimation rate as shown in Equation 2.1. This effect results in the characteristic conical shape observed within the vessels. It would be interesting to find out if there is a temperature gradient within a vial from the bottom to the top during freeze drying. This would probably necessitate a freeze dryer with an infra red transparent door.

In conclusion one can say that when scaling up from a microwell to a vial, or even from an ampoule to a vial, the surface area in contact with the walls of the vessel must be taken into account. Scale up can be considered in terms of constant

volume or constant fill depth. There is much scope for further work, particularly with a real product that forms a porous plug on top of the receding ice front. Such a plug would affect the sublimation rate. With water, the sublimation rate was linear but one would expect the sublimation rate of a product to steadily decrease as the dried layer increases. This is due to the resistance to mass flow caused by the dried layer. The thickness of the layer is increasing with time, but the rate at which it increases is dependent on the sublimation rate. To date, attempts to measure the moisture content of freeze-dried materials in microwells using Karl Fischer titration have been unsuccessful (data not shown). The need to weigh the quantity of material within the well accurately has proved difficult. There now exists a technique of moisture analysis called thermo-gravimetric analysis. In this method, a pan approximately 5 mm in diameter is weighed and then filled with the freeze dried material under nitrogen in a dry box. It is then sealed and weighed again to give the mass of material under test. The pan is then pierced and heated and the gasses released are run through a mass spectrometer. The mass spectrometer allows the user to identify the moisture content present in the sample and differentiate it from any water released due to thermal decomposition. Due to the quantities used, such a method potentially lends itself well to ultra scale-down.

It can further be concluded that the presence of foreign objects such as thermocouples in vials during freeze drying conduct heat into the ice and will therefore interfere with the lyophilization within that vial. As such, thermocouple analysis is not representative of all the vials undergoing lyophilization.

Although microwells would never be a perfect mimic for vials due to the geometric constraints such as the thickness of the vessel wall and indeed the material which the wall is made out of, through the use of thermo gravimetric analysis one could screen a process design and identify maximum shelf temperatures and minimum chamber pressures outside which the run could fail through cake collapse or blowout.

In terms of the aims of this thesis, aside from the thermo gravimetric analysis which has only recently become available, it was decided not to use ultra scale-down to predict drying cycles, due to the inability to determine how dry a sample in a microwell is. However, in terms of formulation screening and optimisation, this investigation showed that it is possible to freeze dry samples within microwells. Therefore the next step was to combine ultra scale-down with factorial design to identify optimum formulations for freeze-drying proteins.

# Chapter 3. Rapid Optimisation of Protein Freeze-Drying Formulations Using Ultra Scale-Down and Factorial Design of Experiment in Microplates[1]

## 3.1 Introduction

Having established that it is possible to freeze dry material in microtitre plates this investigation looks at the use of ultra scale down to identify optimum formulations for freeze drying. Due to the ever increasing complexity and costs of manufacture of biopharmaceuticals, there exists a need for the development of a rapid screening technique that allows the user to find an ideal formulation for the preservation of proteins during freeze-drying, while minimising the quantity of precious materials used. The formulation and process parameters for freeze-drying must maintain the protein stability both during the process and in subsequent storage (31, 72). Freeze-drying must also result in a product with an elegant cake structure, no signs of meltback or collapse, and a low residual moisture content (31). Although much is currently understood about the process and the mechanisms by which excipients such as PEG and sugars protect proteins during freezing and drying (79, 80), an empirical approach is generally used for new protein products (72). A basic formulation design is typically taken as a starting point, where buffers, bulking agents and stabilizers are chosen using as much scientific knowledge as possible about the protein product. This process

[1] Grant Y, Matejschuk P, Dalby P. Rapid optimization of protein freeze – drying formulations using ultra scale – down and factorial design of experiment in microplates Biotechnol. Bioeng. 104(5), 957-964 (2009)

can be time consuming, material intensive and costly due to the hit and miss approach taken and the number of repeats required to validate the results.

As mentioned earlier in the introduction, the use of factorial design of experiment (DoE) software is becoming increasingly popular in industry for screening and optimising processes. Here we combine the use of statistical experimental design with ultra scale-down methods in a microplate format to optimise the formulation for stability to freeze-drying, using small quantities of protein.

The enzyme lactate dehydrogenase (74) was chosen as a suitable model protein to investigate the USD methodologies as it was known to be inactivated during lyophilization (79). Furthermore LDH has been used in the past as a model protein in lyophilisation experiments (73, 74) and has been well characterised in terms of protein structure (81) and protein subunit dissociation (82). Lactate dehydrogenase catalyses the interconversion of pyruvate and lactic acid using NADH as a cofactor, thus also providing a simple assay amenable to use in microplates similar to the procedure described by Krieg et al (83).

### 3.2 Materials and Methods

PEG (MW 2000), Dextran, Glycine, Sodium Pyruvate, Sodium Phosphate (monobasic and dibasic), trehalose, and Lactate Dehydrogenase *(*from *Lactobacillus leichmanii)* were all purchased from Sigma-Aldrich Chemical Co (Poole, UK). NADH was purchased from Calbiochem (Nottingham, UK), lactose and arginine from VWR (Leicestershire, UK), sucrose from FSA (Loughborough, UK). LDH (282 U/mg) was purchased as a lyophilized powder and was

resuspended for baseline assaying in 100mM phosphate buffer at pH 7.0 or for lyophilization in 50mM phosphate buffer at pH 7.0 and diluted to the desired concentration. The 96 well microplates were purchased from Greiner (Greiner, Stonehouse UK) and the bottom lips trimmed off such that the bottom of the wells rested directly on the freeze drier shelves.

### 3.2.1 Determination of edge effects for sublimation from a microplate

Into each of the 96 wells of 3 microplates was pipetted 200 µL of water and then frozen at -20$^{o}$C. The plates were then partially freeze-dried under vacuum at -20$^{o}$C for 90 mins. The remaining ice was then thawed and measured volumetrically for each well using a 200 µL gas-tight Hamilton syringe. The results from each well on each plate were averaged and plotted in Figure 3.1.

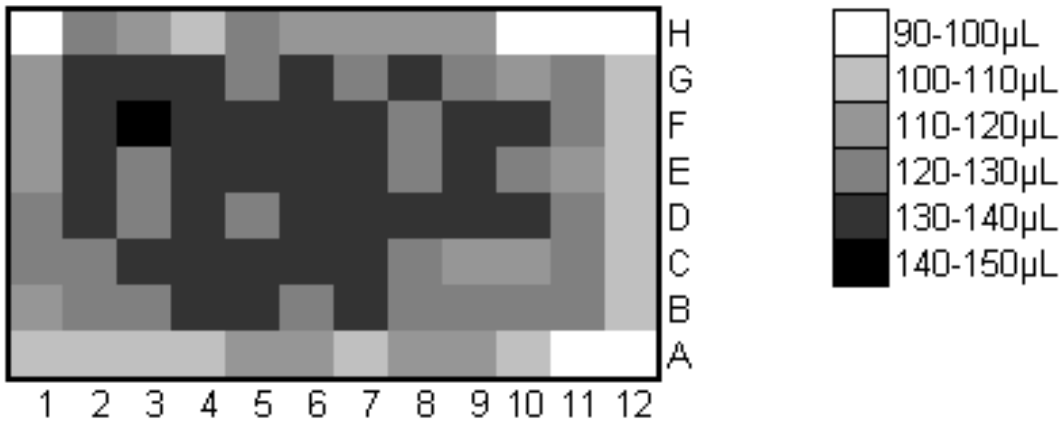


**Figure 3.1 Distribution of the remaining volumes (µL) of water present in each well of a microplate, after partial freeze-drying of 200 µL at 20 $^{o}$C for 90 mins. All points represent an average that had a variability of 5%.**

### 3.2.2 Lactate dehydrogenase activity measurement in microplates

LDH activity retention was measured as a percentage of the original activity. Initial activity was determined for the conversion of pyruvate to lactic

acid. The total powder in a vial of LDH containing 52.4% protein in 3.4-3.5mg solid at 282 U/mg was dissolved in 5 ml 100 mM phosphate buffer, pH 7.0 for a stock concentration of 100 U/ml. A solution of 0.5 U/ml LDH was made from 100 µL stock solution and 19.9 ml 100 mM phosphate buffer, pH 7.0. To each well of a flat bottomed 96-well Greiner microplate (Greiner, Stonehouse UK) was added 20 µL of the 0.5 U/ml LDH and 180 µL of each excipient combination with NADH in 100 mM phosphate buffer, pH 7.0, giving final concentrations of 0.05 U/ml LDH and 0.5 mg/ml NADH. Excipient concentrations were varied as specified in Table 3.1 and Table 3.2. Six replicates per formulation were incubated for 20 mins to allow equilibration of the NADH into the LDH. To initiate reactions, 100 µL of 10 mg/ml sodium pyruvate in 100 mM phosphate buffer, pH 7.0 was added and the absorbance at 340 nm measured in a Versamax Plate Reader (Molecular Devices, Wokingham UK) every 30 seconds for 20 minutes. The absorbance was measured for 20 mins and plotted on a chart. The decrease in absorbance over the first four minutes was always linear. The gradient of the graph over the first four minutes was used to identify the initial activity of the LDH.

| Run Order | Arginine (%w/v) | PEG (%w/v) | Lactose (%w/v) | Trehalose (%w/v) | Sucrose (%w/v) | Dextran (%w/v) | Glycine (%w/v) |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 2 | 0 | 0.5 | 0.5 |
| 2 | 0.5 | 0 | 0 | 2 | 2 | 0.5 | 0 |
| 3 | 0.25 | 0.25 | 1 | 1 | 1 | 0.25 | 0.25 |
| 4 | 0.5 | 0.5 | 2 | 2 | 2 | 0.5 | 0.5 |
| 5 | 0.5 | 0.5 | 2 | 0 | 2 | 0 | 0 |
| 6 | 0 | 0.5 | 0 | 0 | 2 | 0.5 | 0 |
| 7 | 0 | 0 | 2 | 2 | 2 | 0 | 0 |
| 8 | 0 | 0.5 | 2 | 2 | 0 | 0.5 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0.25 | 0.25 | 1 | 1 | 1 | 0.25 | 0.25 |
| 11 | 0 | 0.5 | 2 | 0 | 0 | 0 | 0.5 |
| 12 | 0.5 | 0.5 | 0 | 2 | 0 | 0 | 0 |
| 13 | 0 | 0.5 | 0 | 2 | 2 | 0 | 0.5 |
| 14 | 0.5 | 0 | 0 | 0 | 2 | 0 | 0.5 |
| 15 | 0.5 | 0 | 2 | 0 | 0 | 0.5 | 0 |
| 16 | 0.5 | 0 | 2 | 2 | 0 | 0 | 0.5 |
| 17 | 0.5 | 0.5 | 0 | 0 | 0 | 0.5 | 0.5 |
| 18 | 0 | 0 | 2 | 0 | 2 | 0.5 | 0.5 |
| 19 | 0.25 | 0.25 | 1 | 1 | 2 | 0.25 | 0.25 |

**Table 3.1 Percentages by weight of excipients present in initial screening design formulations for freeze-drying of LDH in microplates**

| Run Order | PEG (%w/v) | Lactose (%w/v) |
|---|---|---|
| 1 | 0.25 | 1 |
| 2 | 0.25 | 1 |
| 3 | 0.5 | 1 |
| 4 | 0.5 | 2 |
| 5 | 0.25 | 1 |
| 6 | 0.25 | 0 |
| 7 | 0.25 | 2 |
| 8 | 0.5 | 0 |
| 9 | 0 | 2 |
| 10 | 0 | 1 |
| 11 | 0 | 0 |

**Table 3.2 Percentages by weight of excipients present in the DoE optimisation formulations for freeze-drying of LDH in microplates**

### 3.2.3 LDH freeze-drying and activity measurement in microplates

LDH (0.05 U/ml) was prepared as above in 200 µL aliquots of 50 mM phosphate buffer pH 7.0, containing the relevant excipient combinations, with 6 wells per formulation. The position of each formulation in the microplate was

randomized by the Design Expert v7 software (Stat-Ease, Minneapolis, USA). Due to potential edge effects all outside edge wells were filled only with buffer. Microplates were placed in a Virtis Genesis 25EL freeze dryer (Biopharma Process Systems, Winchester, UK) and the samples freeze dried under the conditions shown in Table 3.3. The cake was then re-suspended in 200 μL 50 mM phosphate buffer containing 0.5 mg/ml NADH bringing the total concentration of phosphate buffer to 100 mM. Following a 20 minute incubation period, 100 μL of 10 mg/ml sodium pyruvate in 100 mM phosphate buffer was added and the activity measured as above.

| Step | Temperature (Celsius) | Time (mins) | Vac (mTorr) |
|---|---|---|---|
| Freeze | -40 | 120 | |
| Primary Drying | -40 | 600 | 75 |
| Secondary Drying | 20 | 300 | 75 |

**Table 3.3 Lyophilization cycle used for ultra scale-down experiments in microplates**

A fractional factorial design was initially used to identify the effects of seven excipients on the preservation of LDH activity during freeze drying. The seven excipients chosen were selected based on their suitability as FDA approved additives, and on their use in previous protein formulation studies. Concentration ranges were set to indicate likely lower and upper concentrations derived from such studies (102). The concentration ranges used for these excipients are shown in Table 3.4. The design matrix for the fractional factorial screen is shown in Table 3.5 and is presented in the randomised order used. Each condition was measured once, except the condition with all excipients at their midpoint values which was performed in triplicate. This design matrix provides eight conditions

for both the high and low concentrations of each excipient. The measured effect of a factor is therefore a measure of the average effect caused when that factor switches from its low value to high value. As such, in this experiment the measured effects for each factor are the results of eight repeat experiments.

| Name | Experimental Range |
|---|---|
| Sucrose | 0% - 2% |
| Trehalose | 0% - 2% |
| Lactose | 0% - 2% |
| PEG | 0% - 0.5% |
| Dextran | 0% - 0.5% |
| Glycine | 0% - 0.5% |
| Arginine | 0% - 0.5% |
| | |

**Table 3.4 Excipients and concentration ranges investigated in the factorial screening and DoE optimization experiments**

| | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 | Factor 6 | Factor 7 |
|---|---|---|---|---|---|---|---|
| Run | A:Arginine | B:PEG | C:Lactose | D:Trehalose | E:Sucrose | F:Dextran | G:Glycine |
| 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 |
| 2 | -1 | -1 | 1 | -1 | 1 | 1 | 1 |
| 3 | 1 | -1 | 1 | 1 | -1 | -1 | 1 |
| 4 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 5 | 1 | -1 | -1 | -1 | 1 | -1 | 1 |
| 6 | -1 | 1 | 1 | 1 | -1 | 1 | -1 |
| 7 | -1 | -1 | -1 | 1 | -1 | 1 | 1 |
| 8 | 1 | 1 | -1 | -1 | -1 | 1 | 1 |
| 9 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 10 | 1 | 1 | 1 | -1 | 1 | -1 | -1 |
| 11 | -1 | 1 | -1 | -1 | 1 | 1 | -1 |
| 12 | 1 | 1 | -1 | 1 | -1 | -1 | -1 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 1 | -1 | -1 | 1 | 1 | 1 | -1 |
| 15 | -1 | -1 | 1 | 1 | 1 | -1 | -1 |
| 16 | -1 | 1 | -1 | 1 | 1 | -1 | 1 |
| 17 | -1 | 1 | 1 | -1 | -1 | -1 | 1 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3.5 Design matrix for the factorial screening investigation of excipient effects on retention of LDH activity after freeze-drying in microplates. A value of -1 represents the lowest concentration setting for each factor, 1 the highest and 0 the midpoint**

The experiments for optimisation of PEG and lactose excipient combinations in microplate freeze-drying were designed in Design Expert v7 using random surface modelling and a central composite face design, based on the results from the initial fractional factorial investigation. All factors are varied at three levels, high middle and low, to accurately develop a model including any curvature. The design matrix for the optimisation is shown in Table 3.6.

| | Factor 1 | Factor 2 |
|---|---|---|
| Run | A:PEG | B:Lactose |
| 1 | 0 | 0 |
| 2 | -1 | 0 |
| 3 | -1 | 1 |
| 4 | -1 | -1 |
| 5 | 0 | 0 |
| 6 | 0 | -1 |
| 7 | 1 | -1 |
| 8 | 0 | 1 |
| 9 | 0 | 0 |
| 10 | 1 | 1 |
| 11 | 1 | 0 |
| | | |

**Table 3.6 Design matrix for the DoE optimisation investigation of excipient effects on retention of LDH activity after freeze-drying in microplates. A value of -1 represents the lowest concentration setting for each factor, 1 the highest and 0 the midpoint**

### 3.2.4 Verification of optimal freeze-drying formulation in vials

To validate that the formulation selected through the microplate model was appropriate for products lyophilised in vials a scale-up study was performed. The formulations shown in Table 3.7 were made to a fill volume of 1ml in 22mm vials (Adelphi Tubes, Haywards Heath, UK) and then lyophilized using the cycle shown in Table VIII in a Virtis Genesis 25EL freeze dryer (Biopharma Process

Systems, Winchester,UK). The material was then re-suspended and pipetted into microplates to be assayed for activity as described above.

| Formulation | PEG | Lactose | Prediction | -95% | 95% | Actual | Error |
|---|---|---|---|---|---|---|---|
| | % W/W | % W/W | % Retained | % Retained | % Retained | % Retained | +/- |
| 1 | 0.16 | 2 | 74 | 66 | 82 | 75.5 | 2.1 |
| 2 | 0.5 | 0.5 | 75 | 67 | 83 | 75.8 | 1.2 |
| 3 | 0.21 | 0.9 | 75 | 69 | 81 | 78.4 | 0.9 |
| 4 | 0.5 | 2 | 91 | 81 | 101 | 89.6 | 1.9 |
| 5 | 1.7 | 0.41 | 106 | 100 | 112 | 100 | 1.3 |

**Table 3.7 Formulations used for scale up experiments for freeze-drying in 22mm vials. Excipient concentrations are shown as percentage by weight in 50 mM phosphate buffer, pH 7.0. Prediction and actual activity retentions for the formulations are given as a percentage compared to the initial activity**

## 3.3 Results and Discussion

### 3.3.1 Edge effects present on a microplate

The volume of water remaining in each well of a 96-well plate, after partial lyophilization for 90 minutes, as measured using a Hamilton syringe is shown in Figure 3.1. The average standard deviation from plate to plate, of the volume remaining within each well was 5.9 μL. Wells on the edge are shown to have a smaller volume of water remaining inside them after sublimation than the wells in the centre. This would indicate that sublimation was occurring more rapidly in the outer wells and is likely due to thermal transfer via radiation from the sides of the dryer. The complete lyophilization cycle used for the LDH formulations (Table 3.3) was designed to remove all of the ice by sublimation over a period of 600 minutes, leaving a similar final residual moisture content in all wells. However to minimise any potential problems that could occur due to different sublimation rates during DoE experiments, it was decided that the outer

wells should be filled with buffer and only the inner wells used for LDH lyophilization.

### 3.3.2 Fractional factorial design of the effects of excipients on freeze drying

Seven excipients were initially chosen for investigation into their ability to preserve the protein during the freeze drying process and the chosen ranges are described in Table 3.4. These widely used excipients are all generally regarded as safe for use with biopharmaceuticals. The ranges for each excipient were chosen based on typical concentrations used in industrial processes (31, 72). A full factorial design for 7 factors would typically mean 128 individual experiments which would identify all interactions between multiple factors. For the screening investigation a lower resolution was chosen to lower the overall number of experiments necessary while providing sufficient information to identify the main effects and potential 2-factor interactions for further investigation and optimisation. The design matrix for the fractional factorial screen is shown in Table 3.5 using coded values where -1 represents the low value for the excipient concentration (0% w/w), 1 represents the high value for excipient concentration (as shown in Table 3.4) and 0 represents the mid-point value. As shown in Table 3.5 runs 13, 18 and 19 contain all excipients set to their respective mid-point values. This formulation represents the centrepoint of the design space and was repeated three times to allow a measure of accuracy to be determined.

The purpose of these experiments was to rapidly identify which compounds had a positive effect on the maintenance of the stability of lactose dehydrogenase during lyophilization. Data collected from the plate reader assay was plotted on a half normal plot as shown in Figure 3.2. Points on a half normal

plot that deviate significantly from the straight line represent factors that have a significant effect on the retention of activity. In this experiment factor B (PEG 2000) was seen to have a strong effect on activity retention. The point labelled BC also shows a strong effect from a lactose–PEG interaction. It is important to note that as this was a fractional factorial where two-factor interactions were aliased, and so BC could also be AE (arginine – sucrose) or DF (trehalose – dextran). However as PEG was already shown to have an effect on activity retention and none of the other possible factors had a significant effect, it was reasonable to assume that the interaction involved PEG. The other factor in the interaction was lactose so this was included in the subsequent model and optimisation to maintain the hierarchy. While we do not study time-dependent degradations during storage, lactose is known as a reducing sugar and can lead to take part in Maillard reactions which could result in degradation of the product over time in storage. However several biopharmaceutical products currently use it in their formulations to maintain activity (84). Analysis of variance (ANOVA) of the model gave a p-value of 0.0002 showing that the model is statistically significant. This means that the model was sufficiently accurate to determine key factors with potential for optimisation. The model plot in Figure 3.3 shows the interaction between lactose and PEG. At lower concentrations of PEG, increasing the concentration of lactose results in an increase in activity retention of 24% whereas at higher concentrations of PEG the 95% confidence intervals overlap suggesting that statistically there should be no difference. Also, the intersection of the two lines is not on the centre points at 0.25% PEG, suggesting that a linear model is inaccurate and that there should be some degree of curvature in the

model. Therefore determination of the optimum concentrations of excipients required a higher level factorial experiment.

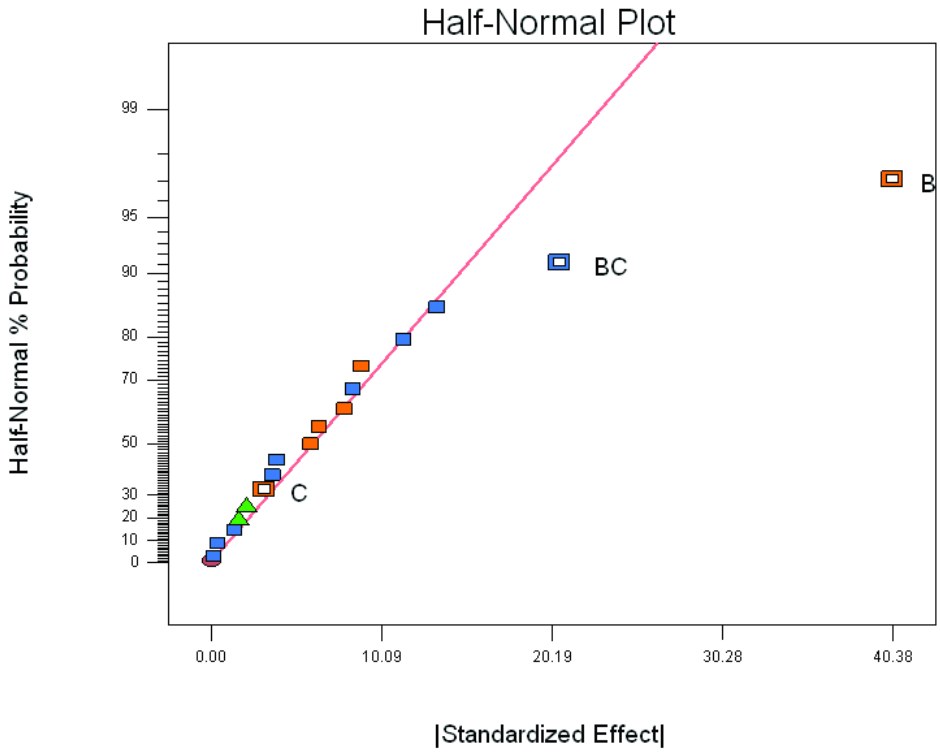


**Figure 3.2 Half normal probability plot for the effects of excipient combinations upon retention of LDH activity during freeze-drying in microplates. B represents PEG 2000 and BC represents the interaction between PEG 2000 and lactose. Orange points demonstrate a positive effect while blue points represent a negative effect and green triangles represent centre point experiments**

### 3.3.3 Central Composite Face Designed Experiment for Optimisation Investigation

Following analysis of results from the fractional factorial, PEG and lactose were identified as key factors for further investigation and optimisation. Using random surface modelling a central composite face design was employed to

identify the optimal formulations based on the results identified in the initial screening investigation. In this experimental design, all factors were varied at three levels, high middle and low, to accurately develop a model which would also include any curvature. The design matrix for the optimisation is shown in Table 3.6 with the coded values shown in Table 3.5. Runs 1, 5 and 9 contained identical formulations and represent the centrepoints of the design space investigated. The results from these experiments were used to develop a quadratic model of the system. The accuracy of the model was assessed using ANOVA and the p-value was determined to be 0.0001 which again shows the model is statistically significant. Therefore, any conclusions based on the results and the model could be considered valid and accurate. The results are plotted on the surface graph shown in Figure 3.4 which demonstrates the curvature present in the model. It also shows that contrary to the initial conclusion based on Figure 3.3, the optimum formulation involves the presence of lactose as well as PEG, for example, 2% lactose and 0.5% PEG will give slightly less retention of LDH activity than at 1.8% lactose and 0.4% PEG. Thus the addition of excess PEG and/or lactose can actually be detrimental to maintaining LDH stability. The plot shown is based on the model described in the following equation which can be used to find the optimum solution:

Percentage Activity Retained = -64.25 +548.6[PEG] +88.32[Lactose] -32.26[PEG][Lactose] -659.1$[PEG]^2$ -24.69$[Lactose]^2$

Where [PEG] and [Lactose] are measured as percentage concentrations on a weight by weight basis.

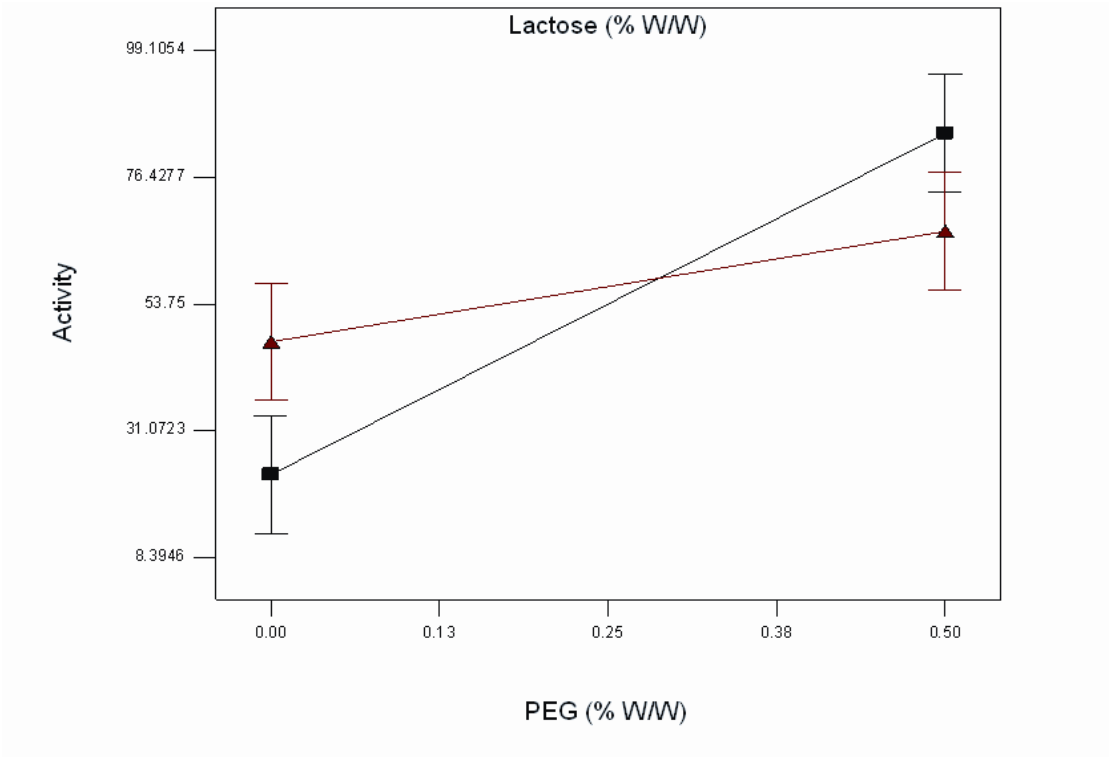


**Figure 3.3 Interaction graph from fractional factorial screening investigation showing combined effect of increasing the concentration of PEG 2000 from 0%-0.5% with lactose concentration set at 0% (black line) and 2% (red line) upon retention of LDH activity during freeze-drying in microplates, where all other factors are set to 0% concentration. The confidence intervals are set to 95% and demonstrate the region where one could expect to see the result appear 95% of the time. However the mean of a range of experiments would lie in the centre of the intervals.**

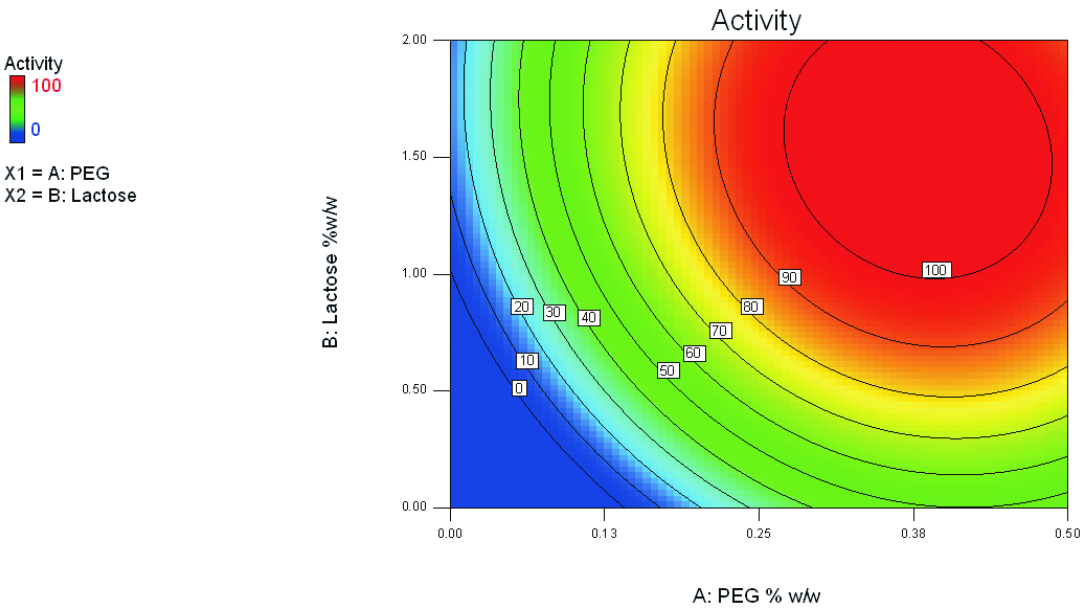


**Figure 3.4 Surface plot showing the effect of varying PEG 2000 (x-axis) and lactose (y-axis) concentrations, upon the retention of LDH activity (z-axis) after freeze-drying.**

Luthra et al (85) have shown recently that LDH can be stabilised by lyoprotectants which promote hydrogen bonding or which form good glassy states. Lactose would be expected to demonstrate the former. PEG has also been shown to maintain stability through cryoprotection of LDH during the freezing stage (75).

During the freeze-thawing of proteins, PEGs are thought to act as a cryoprotectant through several potential mechanisms that also depend on the PEG molecular weight. For LDH these included non-specific binding of PEG to the protein, and delayed ice crystallization during freezing, but did not include their preferential exclusion from the protein surface, or the suppression of protein aggregation (75). Non-specific binding of PEG to the protein surface could provide a protective shell that minimises protein:protein interactions, but it may

also result in a more ordered water structure around the protein which would reduce the nucleation of ice close to the protein surface (86). During the resuspension of the lyophilised protein PEGs could potentially also act as chaperones that favour folding to the native state for partially denatured proteins, in addition to directly stabilising the native state as is often seen for proteins in solution (76).

### 3.3.4 Verification of Optimal Freeze-Drying Formulation in Vials

Having identified a region of optimum formulations using experiments in microplates it was important to verify that the predictions made by the model were verified upon scale up to vials. The vials containing the formulations shown in Table 3.7 underwent the lyophilization cycle shown in Table 3.8. Table 3.7 shows the predicted activity retention by the model together with the 95% confidence intervals. The results were all close to the predicted mean and within the predicted confidence intervals thus verifying the accuracy of the model.

| Step | Temperature (Celcius) | Time (mins) | Vac (mTorr) |
|---|---|---|---|
| Freeze | -40 | 120 | |
| Primary Drying | -40 | 1200 | 75 |
| Secondary Drying | 20 | 600 | 75 |

**Table 3.8 Lyophilization cycle used for pilot scale freeze-drying in 22mm vials.**

### 3.4 Conclusions

The purpose of this investigation was to develop a generic framework for the rapid identification of an optimum formulation for the purposes of maintaining activity during a lyophilization process. It further aimed to show the benefits of using microwells for optimisation in order to minimize the quantity of material

needed to carry out sufficient experiments. In order to conduct a 2 level investigation of 7 excipients followed by a 3 level investigation of 2, one would have to conduct 27+32=137 experiments using an OFAT approach, which including repeats would have used grams of material and would have been time consuming. Furthermore without appropriate statistical analysis it would not be possible to identify interactions. The approach detailed here required a total of 30 experiments which is a reduction of approximately 22% and used approximately 13 micrograms of LDH.

This investigation identified lactose and PEG as effective excipients in maintaining LDH stability during freeze drying. Lactose is thought to stabilise LDH through the promotion of hydrogen bonding (85). PEGs can act as a cryoprotectant for LDH by delaying ice crystallization during freezing, and also by forming non-specific interactions with the protein that then minimise protein:protein interactions (75), while also reducing the nucleation of ice close to the protein surface (86).

The DoE model generated in this study predicted a formulation which retained almost 100% of the activity, and was then shown to be correct on scale up to vials. Were this method to be adopted in an industrial process one could develop a formulation and drying cycle more quickly than by current methods. Furthermore, the model could be used for process validation to determine the upper and lower limits of the operating parameters.

# Chapter 4. Rapid Identification and Optimisation of Freeze Drying Formulation for Granulocyte Colony Stimulating Factor

## 4.1 Introduction

The previous chapter demonstrated how factorial design can be combined with ultra scale down to provide a rapid screening and optimisation platform technology to identify a formulation for the lyophilization of biopharmaceuticals. The protein under investigation was lactate dehydrogenase, a model protein that is reasonably stable under normal conditions. It was therefore necessary to apply this platform to a challenging protein that is used as a therapeutic and is known for its instability and difficulty to freeze dry, so that the general applicability of the techniques can be demonstrated.

Granulocyte colony stimulating factor (G-CSF) is a therapeutic protein marketed under the brand name Neupogen. Chemotherapy can result in neutropenia, a haematological disorder characterised by an abnormally low number of neutrophils in the blood stream. Human G-CSF can be manufactured recombinantly as a non-glycosylated form in *E. coli* which is effective in treating patients suffering from neutropenia (87). Recombinant Human G-CSF (RhG-CSF) is a monomeric cytokine consisting of a four alpha-helical bundle (88, 89) and whose conformational stability in solution is greatest at pH 4.0, but which aggregates within days at pH 7.0 (88). Consequently, RhG-CSF is typically formulated as a liquid only at pH 4.0 and injected directly into patients. Typical liquid formulations for G-CSF at up to 500 μg/ml include acetate or succinate buffer at pH 4.0, a stabilising detergent such as Tween 20 or Tween 80, osmolytes

such as sorbitol, mannitol or trehalose, and human serum albumin as a bulking agent to block and prevent interactions of the G-CSF with vial surfaces (90, 91).

The difficulties associated with freeze drying solutions at pH 4.0 such as concentration effects and pH shifts contribute towards the aggregation of G-CSF during lyophilization and the observed loss of activity on resuspension. There is evidence that the glycosylated eukaryotic expressed material may be successfully lyophilised (92). The therapeutic is marketed as a liquid formulation (93) and stored at 2 – 8 $^{o}$C. Liquid formulations are generally preferred in industry as they provide the product in the form in which it will be used and remove potential errors from resuspension protocol variations.

Biological medicines differ from small molecule APIs in that the materials are not capable of complete characterisation by chemical means alone and so potency is often assigned by reference to a recognised reference preparation. Such materials are typified by the WHO International Standards (www.who.int/biologicals). Many of these materials are lyophilized to facilitate distribution and their GCSF standard 88/646 that is lyophilised is a glycosylated eukaryotic form. There is a requirement for companies to stock standards of their products for quality control purposes. These standards are used as the baseline for all assays conducted on product material. As such, a standard does not need to have a therapeutic concentration at dosage levels for the active protein but rather a concentration at which an appropriate assay can accurately measure its activity. This investigation looks at applying the methods from the previous chapter to a challenging therapeutic protein to develop a lyophilized biological standard of

bacterially expressed G-CSF. Such a standard must maintain biological activity throughout the lyophilization process and then maintain its activity on long term storage. Finally, the formulation should preserve the G-CSF in its unaggregated state for a reasonable period of time upon resuspension into an assay buffer such as PBS at pH 7.0.

## 4.2 Materials and Method

### 4.2.1 Materials

Trehalose, Phenylalanine, media, Sodium Phosphate (monobasic and dibasic) and L-Histidine were all purchased from Sigma-Aldrich Chemical Co (Poole, UK). Mannitol and Arginine were purchased from VWR (Leicestershire, UK), Sucrose from FSA (Loughborough, UK), Tween 20 from Bio-Rad Laboratories (Hercules CA, USA) and Human Serum Albumin (20% w/w) (HSA) was obtained from Grifols (Barcelona, Spain). G-CSF was provided by NIBSC supplied to them as a biosimilar for research/evaluation purposes from an Indian biopharmaceuticals company and was obtained in solution at 1mg/ml at pH 4.0. RPMI – 1640 Media for the cell based assay was purchased from Sigma – Aldrich Chemical Co (Poole, UK) and the tritiated thymidine was purchased from Perkin Elmer (Boston, MA, USA). The assay was carried out using a GNFS – 60 cell line. The 96 – well microplates were purchased from Greiner (Greiner, Stonehouse UK) and the bottom lips trimmed off such that the bottom of the wells rested directly on the freeze drier shelves.

### 4.2.2 GNFS-60 bioassay

GCSF activity was assayed using an in-vitro cell growth activation assay. Serial dilutions of G-CSF were prepared and cell growth was measured by scintillation count of tritiated thymidine as has already been described (112, 113). Each sample was assayed in duplicate on a microtitre plate and compared to a control sample of G-CSF which had not undergone lyophilization. Solutions containing G-CSF were diluted to 10 ng/ml G-CSF in fermentation media. 160 µL media were pipetted into each well in the top row of a 96-well microtitre plate. The rest of the wells on the plate were filled with 100 µL media. 40 µL of solution containing 10 ng/ml G-CSF was then pipetted into the top well giving a final concentration of G-CSF in the top row of 1ng/ml. A serial dilution was then performed down the plate so that subsequent rows contained 500, 250, 125, 63, 31, 16, and 8 ρg/ml G-CSF. 100 µL of media containing $10^5$ cells/ml was then added to each well and the plate was incubated at 37 $^0$C for 48 hours. Each well was then pulsed with 50 µL tritiated thymidine and media and incubated for a further 4 hours at 37 $^o$C. The plate was then harvested onto a filter mat, dried and sealed in a plastic bag containing a scintillant. This was then counted on a Triluz 1450 Microbeta Scintillation Counter (Perkin Elmer, Massachusetts USA) to give a final cell count. This count was plotted and parallel line analysis was then conducted using proprietary software to give a relative potency of the sample under test relative to the untreated G-CSF.

### 4.2.3 G-CSF freeze-drying and activity measuring in microplates

As described in the previous chapter, a fractional factorial design was used to screen seven excipients to identify which excipients best preserve G-CSF

during freeze drying. A categorical factor – buffer type – was also included to assess which buffer performed best or whether a buffer was even necessary. The ranges for the excipient concentrations investigated are shown in Table 4.1. G-CSF (100 ng/ml) was prepared in 200µL aliquots containing the excipients and buffers shown in Table 4.2. Due to potential edge effects all outside edge wells were filled only with buffer. Microplates were placed in a Virtis Genesis 25EL freeze dryer (Biopharma Process Systems, Winchester, UK) and the samples freeze dried under the conditions shown in Table 4.3.

| Name | Units | Type | Low | High |
|---|---|---|---|---|
| Trehalose | % | Numeric | 0 | 2 |
| HSA | % | Numeric | 0 | 2 |
| Buffer | | Categoric | Phosphate 50mM | Histadine 30mM |
| Manitol | % | Numeric | 0 | 0.5 |
| Sucrose | % | Numeric | 0 | 2 |
| Tween 20 | % | Numeric | 0 | 0.1 |
| Arginine | % | Numeric | 0 | 0.5 |
| Phenylalanine | % | Numeric | 0 | 0.5 |

**Table 4.1 Excipients and concentration ranges investigated in the factorial screening experiments**

| Run | Trehalose | HSA | Buffer | Manitol | Sucrose | Tween 20 | Arginine | Phenyl alanine |
|---|---|---|---|---|---|---|---|---|
| | % | % | | % | % | % | % | % |
| 1 | 2 | 2 | Histidine 30mM | 0.5 | 2 | 0.1 | 0.5 | 0.5 |
| 2 | 0 | 0 | Histidine 30mM | 0.5 | 0 | 0 | 0.5 | 0.5 |
| 3 | 0 | 2 | Histidine 30mM | 0 | 0 | 0.1 | 0 | 0.5 |
| 4 | 0 | 2 | Phosphate 50mM | 0.5 | 0 | 0.1 | 0.5 | 0 |
| 5 | 1 | 1 | Phosphate 50mM | 0.25 | 1 | 0.05 | 0.25 | 0.25 |
| 6 | 2 | 0 | Phosphate 50mM | 0.5 | 2 | 0 | 0.5 | 0 |
| 7 | 2 | 0 | Phosphate 50mM | 0 | 0 | 0.1 | 0.5 | 0.5 |
| 8 | 1 | 1 | Histidine 30mM | 0.25 | 1 | 0.05 | 0.25 | 0.25 |
| 9 | 2 | 2 | Histidine 30mM | 0 | 0 | 0 | 0.5 | 0 |
| 10 | 1 | 1 | Phosphate 50mM | 0.25 | 1 | 0.05 | 0.25 | 0.25 |
| 11 | 0 | 0 | Histidine 30mM | 0 | 2 | 0.1 | 0.5 | 0 |
| 12 | 0 | 0 | Phosphate 50mM | 0 | 0 | 0 | 0 | 0 |
| 13 | 2 | 0 | Histidine 30mM | 0.5 | 0 | 0.1 | 0 | 0 |
| 14 | 0 | 2 | Phosphate 50mM | 0 | 2 | 0 | 0.5 | 0.5 |
| 15 | 2 | 2 | Phosphate 50mM | 0 | 2 | 0.1 | 0 | 0 |
| 16 | 0 | 2 | Histidine 30mM | 0.5 | 2 | 0 | 0 | 0 |
| 17 | 2 | 0 | Histidine 30mM | 0 | 2 | 0 | 0 | 0.5 |
| 18 | 0 | 0 | Phosphate 50mM | 0.5 | 2 | 0.1 | 0 | 0.5 |
| 19 | 2 | 2 | Phosphate 50mM | 0.5 | 0 | 0 | 0 | 0.5 |
| 20 | 1 | 1 | Histidine 30mM | 0.25 | 1 | 0.05 | 0.25 | 0.25 |
| | | | | | | | | |

**Table 4.2 Percentages by weight of excipients present in initial all factorial screening formulations for freeze-drying of G-CSF in microplates**

| Step | Temperature (Celsius) | Time (mins) | Vac (mTorr) |
|---|---|---|---|
| Freeze | -40 | 120 | |
| Primary Drying | -40 | 600 | 75 |
| Secondary Drying | 20 | 300 | 75 |

**Table 4.3 Lyophilization cycle used for ultra scale-down experiments in microplates**

The freeze-dried protein cake was then resuspended in d'$H_2O$ and subsequently diluted into media for assaying as described in section 4.2.2.

Following the initial investigation the optimisation experiment was developed in Design Expert as described in Section 3.3.3 using random surface modelling to produce a central composite faced design where each factor was investigated at three levels. The recipes used are shown in Table 4.4.

| Run | Tween | HSA | pH |
|---|---|---|---|
| | % | % | |
| 1 | 0.25 | 2.5 | 4 |
| 2 | 0.25 | 0 | 4 |
| 3 | 0.5 | 0 | 4 |
| 4 | 0 | 2.5 | 4 |
| 5 | 0.25 | 2.5 | 4 |
| 6 | 0.25 | 2.5 | 4 |
| 7 | 0.25 | 5 | 4 |
| 8 | 0.5 | 5 | 4 |
| 9 | 0.5 | 2.5 | 4 |
| 10 | 0.25 | 2.5 | 4 |
| 11 | 0.25 | 2.5 | 4 |
| 12 | 0 | 0 | 4 |
| 13 | 0 | 5 | 4 |
| 14 | 0.05 | 2.5 | 4 |
| 15 | 0.05 | 2.5 | 4 |
| 16 | 0.05 | 0 | 4 |
| 17 | 0.05 | 2.5 | 4 |
| 18 | 0.05 | 2.5 | 4 |
| 19 | 0.1 | 2.5 | 4 |
| 20 | 0.05 | 2.5 | 4 |
| 21 | 0.05 | 5 | 4 |
| 22 | 0.1 | 5 | 4 |
| 23 | 0.1 | 0 | 4 |
| 24 | 0.5 | 0 | 7 |
| 25 | 0.25 | 1 | 7 |
| 26 | 0 | 2 | 7 |
| 27 | 0.5 | 2 | 7 |
| 28 | 0 | 0 | 7 |
| 29 | 0 | 2.5 | 7 |
| 30 | 0.1 | 2.5 | 7 |
| 31 | 0 | 0 | 7 |
| 32 | 0.05 | 5 | 7 |
| 33 | 0.05 | 2.5 | 7 |
| 34 | 0.05 | 0 | 7 |
| 35 | 0.1 | 5 | 7 |
| 36 | 0.1 | 0 | 7 |
| 37 | 0.05 | 2.5 | 7 |
| 38 | 0.05 | 2.5 | 7 |
| 39 | 0.05 | 2.5 | 7 |
| 40 | 0.05 | 2.5 | 7 |
| 41 | 0 | 5 | 7 |
| 42 | 0.05 | 2.5 | 7 |
| 43 | 0.05 | 2.5 | 7 |

**Table 4.4 Percentages by weight of all the excipients present in the optimisation investigation for freeze drying G-CSF in microplates**

### 4.2.4 Verification of Optimal Freeze-Drying Formulation in Vials

In order to validate the model, three formulations at two pH's were lyophilized and are described in Table 4.5.

| Run | Tween (% v/v) | HSA % w/w) | Solvent | pH | - | Predicted Activity | + |
|---|---|---|---|---|---|---|---|
| 1 | 0.22 | 2.64 | Water | 7 | 81 | 100 | 121 |
| 2 | 0.28 | 3.54 | Water | 7 | 84 | 109 | 137 |
| 3 | 0.17 | 3.51 | Acetate Buffer | 4 | 65 | 73.5 | 83 |

**Table 4.5 Formulation recipes for model validation experiments in 22mm vials**

To further demonstrate that no buffer is required a comparison was made between two identical formulations in different solvents – histidine buffer and water and the recipes are shown in Table 4.6.

| Solvent | Tween (% v/v) | HSA % w/w) |
|---|---|---|
| Histadine | 0.33 | 2 |
| Water | 0.33 | 2 |

**Table 4.6 Recipes used to demonstrate effectiveness of water as solvent in vials**

All experiments were conducted using a 1 ml fill volume and lyophilized using the cycle shown in Table 4.7. Although the concentration of G-CSF was 100 ng/ml throughout it was decided to see whether the optimum formulation was valid at 1 μg/ml as well.

| Step | Temperature (Celsius) | Time (mins) | Vac (mTorr) |
|---|---|---|---|
| Freeze | -40 | 120 | |
| Primary Drying | -35 | 900 | 75 |
| Secondary Drying | 20 | 540 | 75 |

**Table 4.7 Drying recipe for validation experiments in vials**

Finally, it was also necessary to demonstrate a lack of interference between the formulation and the bioassay. Unlyophilized G-CSF was formulated

and then assayed in the same manner, to identify potential differences between formulated and unformulated G-CSF and their impact on the assay itself.

### 4.2.5 Stability of Liquid Formulated G-CSF

The overall aim of freeze – drying biopharmaceuticals is to preserve them until needed by a medical practitioner. As such, although preservation of activity is important, it is also vital to know how long after resuspension the drug remains fit for use. It was decided therefore to investigate the liquid stability of G-CSF over a period of 7 days stored at room temperature. It is common for injectable drugs to be re-constituted in PBS so all formulations were prepared in PBS. One set was formulated and lyophilized, one set was formulated and left to stand and one set was formulated in plain PBS. The formulations were reconstituted or formulated on days 0, 3, 5 and day 7. All were then assayed at the same time on day 7 to ensure that day-to-day variations in the assay, due to differences in cell batches, were eliminated when comparing relative activities.

### 4.2.6 Accelerated Degradation Study of Lyophilised G-CSF Formulations

The purpose of lyophilization is to preserve the biopharmaceutical product in a state that is suitable for long term storage e.g. two years. To determine the long term stability of the freeze dried cake, samples were lyophilized and then stored at -20 $^{o}$C, 4 $^{o}$C, 20 $^{o}$C, 37 $^{o}$C, 45 $^{o}$C and 56 $^{o}$C. Accelerated degradation is a widely used method to rapidly assess the likely stability of biopharmaceuticals (96). These were assayed at 4 month and 6 month intervals and compared to a

lyophilized baseline kept at -70 $^{o}$C to give Erying plots of the Arrhenius equation according to Equation 4.1.

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H}{RT} + \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S}{R}$$

**Equation 4.1 Erying equation where k is the reaction rate constant, T is absolute temperature, delta H is enthalpy of activation, R is gas constant, $k_B$ is the Boltzmann constant, h is Planck's constant and delta S is the entropy of activation.**

### 4.3 Results and Discussion

#### 4.3.1 G-CSF Freeze Drying and Activity Measurement in Microplates

Eight factors were initially chosen for investigation using a Design of Experiment (DoE) approach. Seven (excipient concentrations) were numeric and one (buffer type) was a categorical factor. As with LDH the purpose of this experiment was to rapidly identify which factors had a positive effect on maintaining the biological activity of G-CSF during lyophilization. The seven excipient factors chosen were trehalose, human serum albumin (HSA), mannitol, sucrose, Tween 20, arginine and Phenylalanine as detailed in Table 4.1. These were chosen because all are common excipients used as cryoprotectants and lyoprotectants in the industry, including for G-CSF. Trehalose and sucrose for example are very common excipients and the use of mannitol has been well documented due to its ability to crystallize well under acceptable conditions (97, 98). Likewise, the buffer options chosen were 30 mM Histidine Buffer and 50 mM Phosphate Buffer both at pH 7.0 on the basis that they are two common

buffers used in liquid protein formulations (99). HSA is a plasma protein (100) which has been used in the past both to preserve active pharmaceutical ingredient activity and bulk out the cake (101, 102). Although pH shifts are known to occur during freezing with Phosphate Buffer (102, 103), it was decided that due to the relatively low concentration of buffer compared to the other excipients, such shifts would not cause significant damage and the other components may even protect against damage occurring.

The factorial design chosen was a fractional factorial design where each factor was varied at two levels. Twenty runs were required which included four centre point repeat experiments as detailed in Table 4.2. Each formulation was set up in microplates and freeze-dried as described previously in Chapter 3.2.3. Samples were then resuspended in PBS and assayed for biological function in terms of the stimulation of $10^5$ cells (Section 4.2.2) relative to a standard of unlyophilized G-CSF stored in the supplied formulation at pH 4 and 4 $^{o}$C.

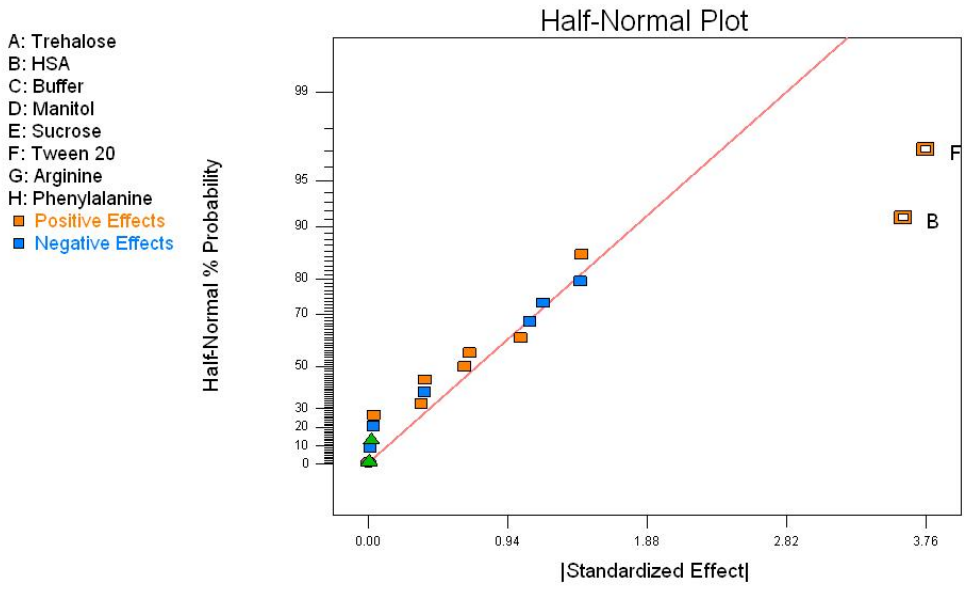


**Figure 4.1 Half Normal Plot of Screening Results**

As described previously (Chapter 3.3.2), a Half-Normal plot is used to identify those factors and interactions that have a significant effect. The Half-Normal plot in Figure 4.1 shows clearly that only two factors influenced the preservation of activity of G-CSF during freeze drying. The normal plot (or Half – Normal plot in this case) is a plot of actual data against predicted data. The prediction is based on the assumption that all factors are non significant and as such the outcomes should be a normal distribution around zero. Therefore any outcome that does not fall within this normal distribution must have a non – zero outcome and must be significant. This is seen by the deviation from the straight line which represents the normal distribution around zero. Here the two most significantly influential factors were HSA and Tween-20. It was interesting to observe that the type of buffer used had no effect at all on the G-CSF as this has often been found to be important for stability at pH 4.0 (104), and also because G-CSF stability is known to be pH sensitive. It is possible that the presence of HSA and/or Tween-20 have some buffering effect such that they were sufficient to negate the requirement for an additional buffer. Clinical grade HSA often contains trace levels of buffer from its preparation and it is commonly used as a stabilizer in protein formulations for a number of reasons. Its amphiphilic properties make it suitable as an additive to inhibit adsorption of the protein onto the wall of the vessel, which due to the low concentration of G-CSF present in these formulations was likely to be important. HSA is also a known lyoprotectant (105). Similarly Tween-20 is also a surfactant and can suppress aggregation of proteins in solution.

Having identified the significant effects, the DoE software was then used to build a model based on these results and the model can be assessed for its

statistical significance through ANalysis Of VAriance (ANOVA). The key figures from ANOVA of the model are the p-value which ideally should be less than 0.05 and the signal to noise ratio or F Value which ideally should be as high as possible. The model has a p-value less than 0.0001 and an F Value of almost 24. Having established the mathematical model is statistically significant it can be used to plot the graph shown in Figure 4.2 which shows the maximum achievable activity preservation is attained at 2% HSA and 0.1% Tween-20. The linear nature of the graph however suggested that an optimum had not been found and that the range of concentrations of HSA and Tween-20 should both be increased. Therefore the ranges were increased up to 5% and 0.5% respectively, for the subsequent optimisation study.

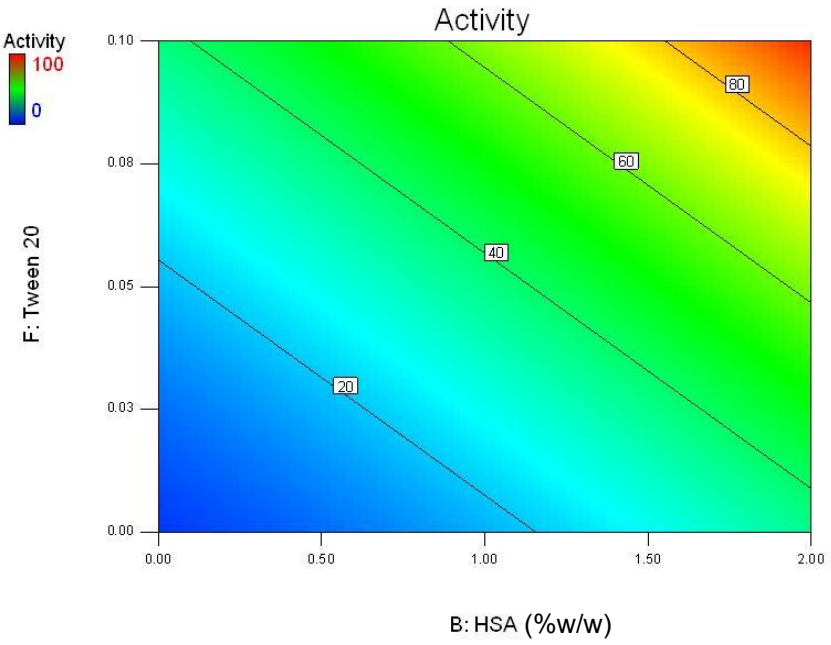


**Figure 4.2 Contour graph showing how activity varies with Tween-20 (%v/v) and HSA concentrations**

The type and addition buffer was shown to be unimportant in terms of the preservation of G-CSF activity, potentially due to the inherent buffering potential of the HSA, Tween-20 or possibly even the G-CSF itself. This can be seen as a serendipitous result as buffers themselves can often cause problems during freeze drying due to pH shifts or salt concentration effects. It was therefore decided to conduct the optimisation study without additional buffer with the final formulations checked to make sure they were at pH 7 (51, 106). However, current commercial liquid formulations for G-CSF are kept at pH 4 at between 2 - 8 $^{o}$C so it was also decided to conduct the optimisation study in water at pH 7 and also for comparison in acetate buffer at pH 4.

Due to the categorical nature of pH as a factor, the results of the optimisation investigation are split into two graphs with one showing formulations dissolved in demineralised water and the other showing formulations dissolved in acetate buffer at pH 4. The final activity results obtained from these two investigations are shown in Figure 4.3 and Figure 4.4 respectively.

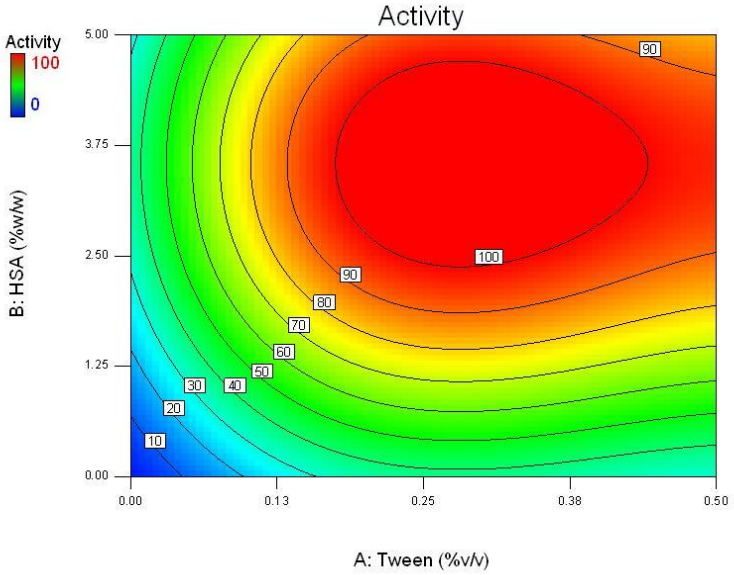


**Figure 4.3 Contour graph showing the effect of varying Tween-20 and HSA on the retention of G-CSF activity in water at pH 7**

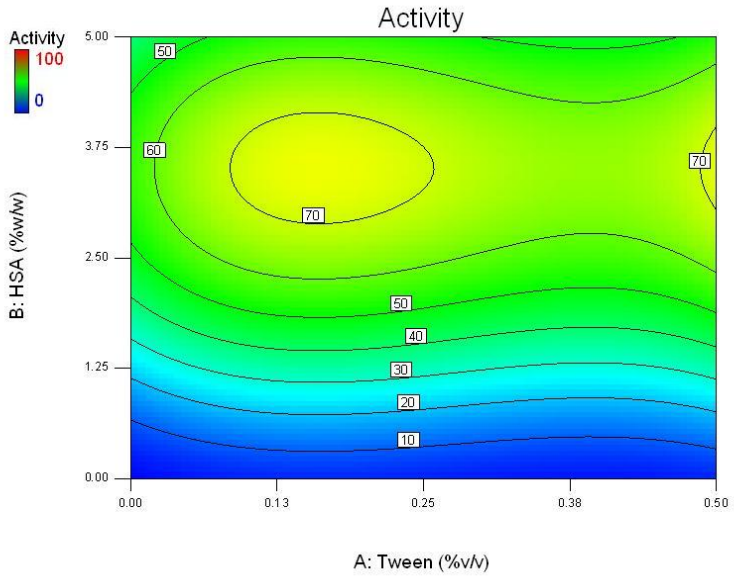


**Figure 4.4 Contour graph showing the effect of varying Tween-20 and HSA on the retention of G-CSF activity in acetate buffer at pH 4**

The response curve shown in Figure 4.3 confirmed the results of the initial screening investigation in that it was now clear that an optimum region existed

outside the range of concentrations for HSA and Tween-20 used initially in the screening experiments. As with LDH excess reagents do not lead to an increase in activity retention but the concentrations must be limited to within the region bounded by the 100% contour. An optimum formulation for G-CSF at 0.1 ug/ml in water at pH 7 was found to be 0.3% (v/v) Tween-20 and 3.5% (w/w) HSA. It is worth noting that no liquid (or freeze-dried) formulations have been previously identified at pH 7, though normally for pharmaceutical applications, the G-CSF is stored as a liquid at higher protein concentrations, typically 100-500 ug/ml).

It is interesting to note that even though G-CSF is most stable in liquid formulation at pH 4 (107). This was not found to be the case during freeze drying at the G-CSF concentration used in this study. It is not possible using Tween-20 and HSA, to achieve 100% retention of activity using a solvent that is at pH 4 initially. This is surprising given that G-CSF is more stable in liquid formulations at pH 4 than at pH 7. The difference in freeze-drying could be due to localized pH shifts and concentration effects, for example when ice crystals form during freezing and exclude the buffer salts, other excipients and G-CSF. Due to such effects and also their additional cost a formulation that does not require a buffer would be advantageous to an industrial bioprocess. At pH 4, an optimum of approximately 70% activity was obtained for 0.15% (v/v) Tween-20 and 3.5% (w/w) HSA, though a second optimum may exist at >0.5% v/v Tween-20 which could potentially achieve greater than 70% retention of activity.

### 4.3.2 Verification of Optimal Freeze-Drying Formulation in Vials

Having identified the optimal region of operation it was necessary to verify that the predictions made by the model remained true on scale up to the types of vials to be used in the creation of freeze-dried biological standards. The formulations for the optimum and a range of sub-optimal conditions described in Table 4.5 were made up to 1 ml per vial, filled and freeze dried using procedure shown in Table 4.7. The results, as shown in Table 4.8, demonstrated a high level of accuracy for the model with the predicted values closely matching the trend for the experimental values, thus verifying the model. The actual experimental results were all within 2% of their predicted values and well within the confidence interval ranges. The model was shown to be highly accurate with the actual results within 2% or even 1% of their predictions in runs 2 and 3. Bearing in mind the variability between different runs of the same assay was often up to 20%, this was a good result.

| Run | Tween | HSA | pH | Actual Result | 95% Confidence Interval Low Value | Predicted Activity | 95% Confidence Interval High Value |
|---|---|---|---|---|---|---|---|
| 1 | 0.22 | 2.64 | 7 | 99.9 | 81 | 100 | 121 |
| 2 | 0.28 | 3.54 | 7 | 97.3 | 84 | 109 | 137 |
| 3 | 0.17 | 3.51 | 4 | 75.6 | 65 | 73.5 | 83 |

**Table 4.8 Formulation recipes and results for model validation experiments**

To further verify that no additional buffer was required for the freeze-drying of G-CSF at pH 7, a comparison was made between two identical formulations in histidine buffer and water as detailed in Table 4.6 (Section 4.2.4). The relative activities retained for samples resuspended after lyophilization, across five repeat vials each containing these formulations, were 110% and 108% for water and histidine respectively. The respective percentage errors were 8% and 7%. It can therefore be concluded that there was no requirement for a buffer

in addition to the excipients present and that the samples, which are at pH 7, must be sufficiently self-buffering at this concentration of G-CSF.

### 4.3.3 Effect of formulation at higher G-CSF concentrations

The aim of this experiment was to identify a formulation suitable for lyophilizing G-CSF for use as a biological standard required at the relatively low concentration of 100 ng/ml. However it would be useful to know whether this formulation had the potential to stabilise G-CSF at the concentrations typically used in pharmaceutical products. Therefore the optimum formulation found here was made again but with 1 μg/ml G-CSF and then lyophilized as previously. This formulation gave 85.5% ± 20.5% activity retention using the cell-based assay, suggesting that the formulation could potentially be sufficient for use in manufacturing G-CSF for medical use.

It is possible that although the concentration of Tween-20 and HSA within the well is extremely low, their concentrations relative to G-CSF remain constant. Whatever protection mechanism these excipients perform on the G-CSF during freeze drying it would appear they continue to protect the protein in liquid too as was investigated further in section 4.3.5.

### 4.3.4 Effect of optimum formulation conditions on activity assays

It was possible that the relatively high levels of Tween-20 in the optimum formulation found above could have interfered with the growth of the cell line in the assay even though the highest concentration of Tween-20 in any well would

not exceed 0.003%. Therefore it was important to verify that the formulation was not having an apparent stabilising effect due to altering the response to the assay itself. To achieve this, two samples were prepared, one sample contained G-CSF at 100 ng/ml in a formulation containing 0.28% Tween-20 and 3.54% HSA in water. The other sample contained G-CSF at 100 ng/ml prepared in RPMI – 1640 Media. Both were then assayed using the same method in Section 4.2.2, and the results can be seen in Figure 4.5.

Although there is a slight difference between the two curves the results show that Tween-20 and HSA do not have any significant effect on the growth of the cells that would account for the optimum formulation effects. The two curves give a relative activity of the formulated to unformulated G-CSF of 110 %, which is much smaller than the difference in activities observed during freeze-drying due to the formulations remaining constant. Whatever protection mechanism these excipients perform on the G-CSF during freeze drying it would appear they continue to protect the protein in liquid too as was investigated further in section 4.3.5.

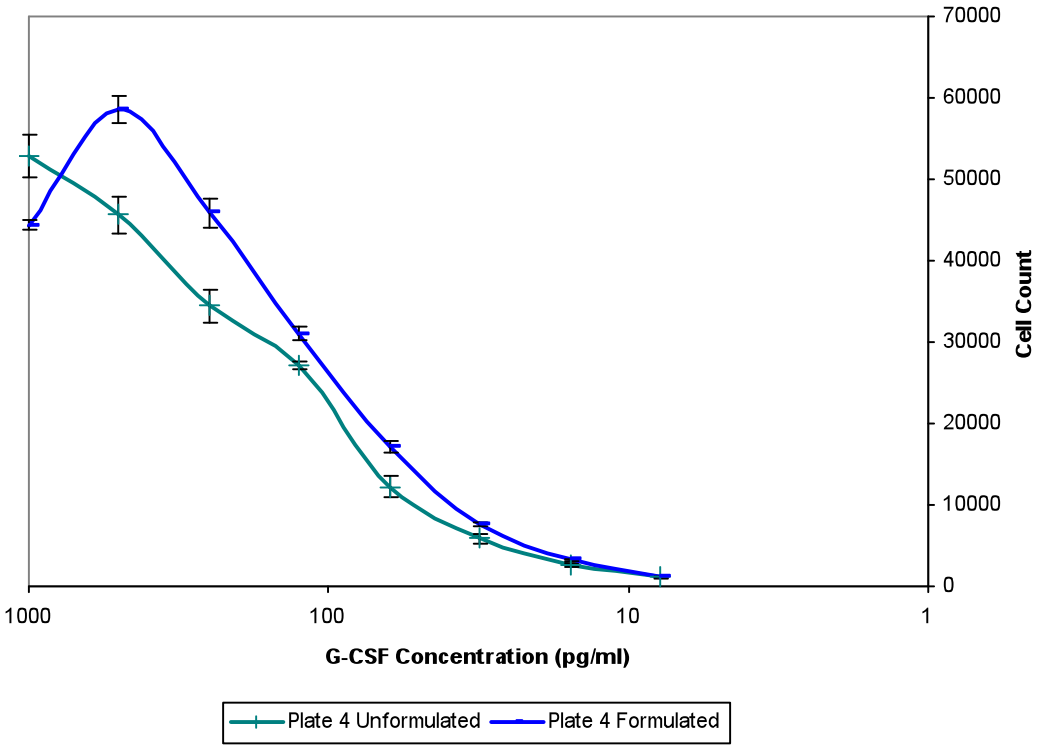


**Figure 4.5 Potency assay curve for formulated and unformulated G-CSF**

### 4.3.5 Liquid stability experiment

Throughout this investigation assaying of the G-CSF was carried out within a short time span of resuspension of the freeze dried solid. The current liquid formulation prevents aggregation of the protein for at least two years at 5 $^{o}$C and even at room temperature the amount of aggregation is minimal. It was necessary to determine if the formulation that maintained activity and structure during freeze drying would do so in solution as well and for how long. It was further necessary to determine whether the protein had been damaged slightly during freeze drying in such a way as to result in a less stable product. Finally an understanding of the stability of the G-CSF in liquid form within this formulation would help develop an understanding of how these excipients protect the protein.

Figure 4.6 is the result of the experiment into the stability of G-CSF in solution. It is immediately apparent that G-CSF in PBS alone is unstable and denatured rapidly. In the time taken to formulate the G-CSF in buffer and take it into the assay it lost 60% of its biological activity. This may however have been due to irreversible binding of the G-CSF to the walls of the vessel in which it was formulated. This problem is overcome through the presence of HSA which preferentially binds to the material either by better binding mechanisms or simply through the higher concentration of HSA relative to the G-CSF.

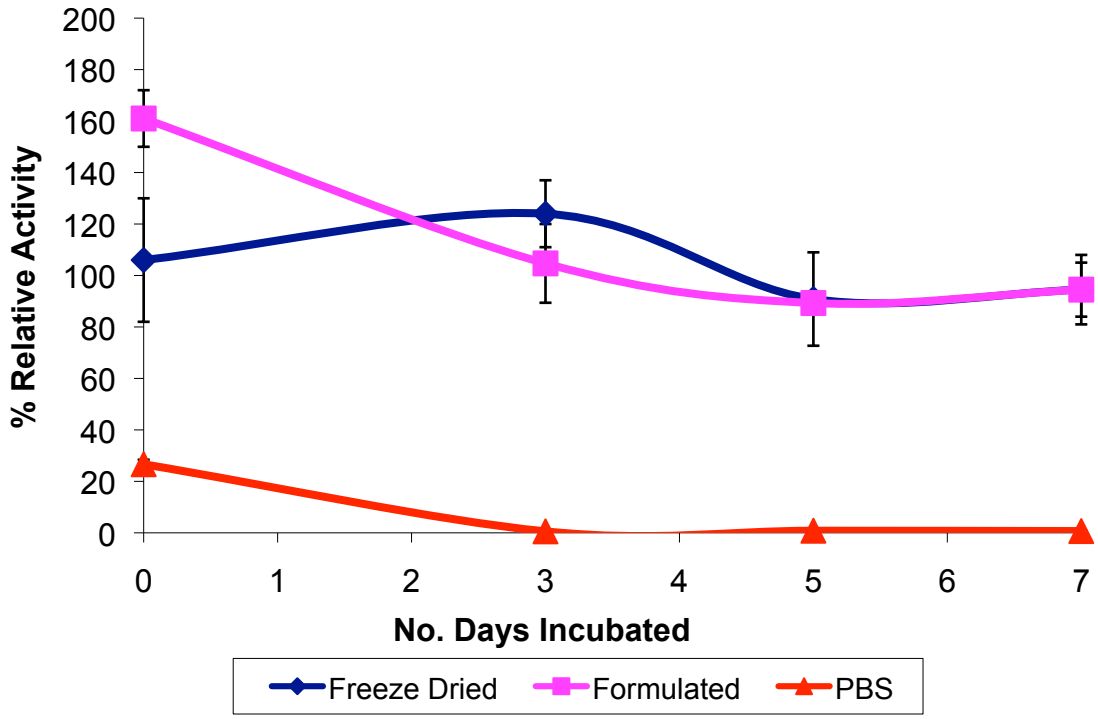


**Figure 4.6 Liquid stability experiment**

This hypothesis could also explain why the relative concentrations of the formulated G-CSF were approximately 60% higher than the control sample taken directly from the liquid formulation. The G-CSF in the control sample is taken from the liquid formulation container at a concentration of 1 mg/ml and diluted to 1 ng/ml in cell media. The G-CSF in the other samples were taken from 1 mg/ml and diluted straight into PBS containing HSA and Tween 20. Any potential binding sites would have been blocked by the HSA. The Tween 20 could then wrap around the protein and prevent aggregation with other protein molecules. The assay is also prone to errors both on behalf of the operator and by the nature of the assay itself so the single unexpected point on the graph can be attributed to error.

One can conclude from Figure 4.6 that while G-CSF in PBS alone aggregated almost immediately and loses all activity within three days, G-CSF in

Tween-20 and HSA can sit on the shelf for a week without losing biological activity even if it was lyophilized first and then resuspended. As such a formulation containing HSA and Tween-20 in concentrations determined using Figure 4.3 can be relied upon to give a stable formulation.

### 4.3.6 Accelerated Degradation Study of Lyophilised G-CSF Formulations

Having developed this systematic approach to optimising biopharmaceutical formulations and having then applied it to the example of G-CSF formulated as a biological standard it was necessary to ensure the formulation is fit for purpose. Here the aim was to have a dried standard that could be kept for two years with minimal loss of activity. As such it was necessary to gather some data regarding the long term shelf life of the finished product. Accelerated degradation studies involve leaving vials of lyophilized material at a range of temperatures in order to assess their stability. Vials were stored at -20 $^{o}$C, 4 $^{o}$C, 20 $^{o}$C, 37 $^{o}$C, 45 $^{o}$C and 56 $^{o}$C. These were then assayed at 4 month and 6 month intervals and compared to a lyophilized baseline kept at -70 $^{o}$C to give Erying plots of the Arrhenius equation. Two lyophilized vials were further assayed immediately after lyophilization to ensure the entire batch still had 100% of their original activity and had not become contaminated during the process (contamination can interfere with the assay and prevent reliable results).

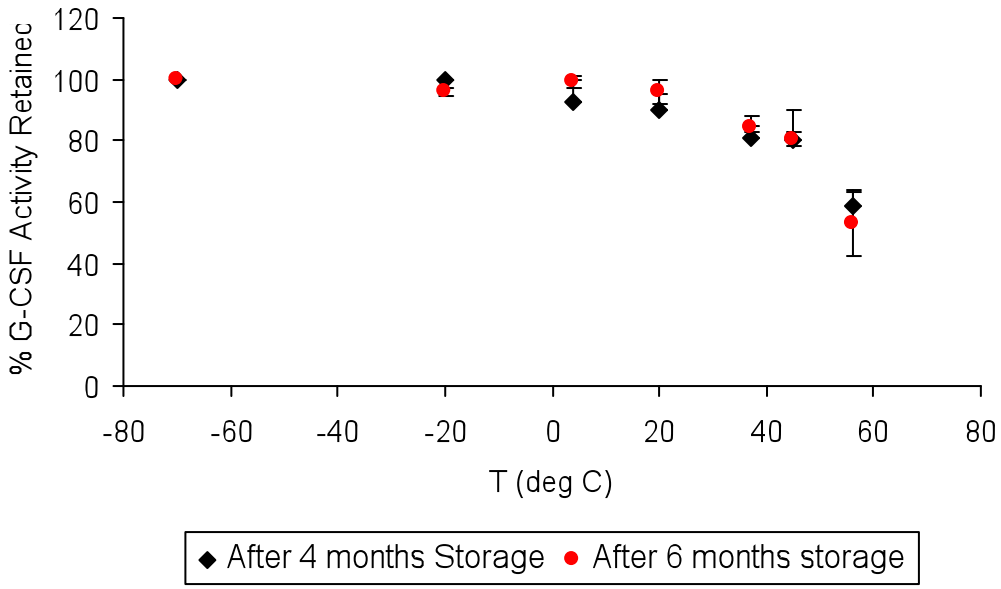


**Figure 4.7 Chart showing percentage activity retained by the freeze-dried formulation at pH 7 after storage at different temperatures**

As Figure 4.7 demonstrates, little activity is lost for temperatures up to room temperature. Thereafter at higher temperatures the protein starts to degrade suggesting that it is quite a heat sensitive labile protein structure. The extent of degradation is not proportional to temperature but rather indicates that the degradation may follow an Arrhenius dependence typical of chemical reactions.

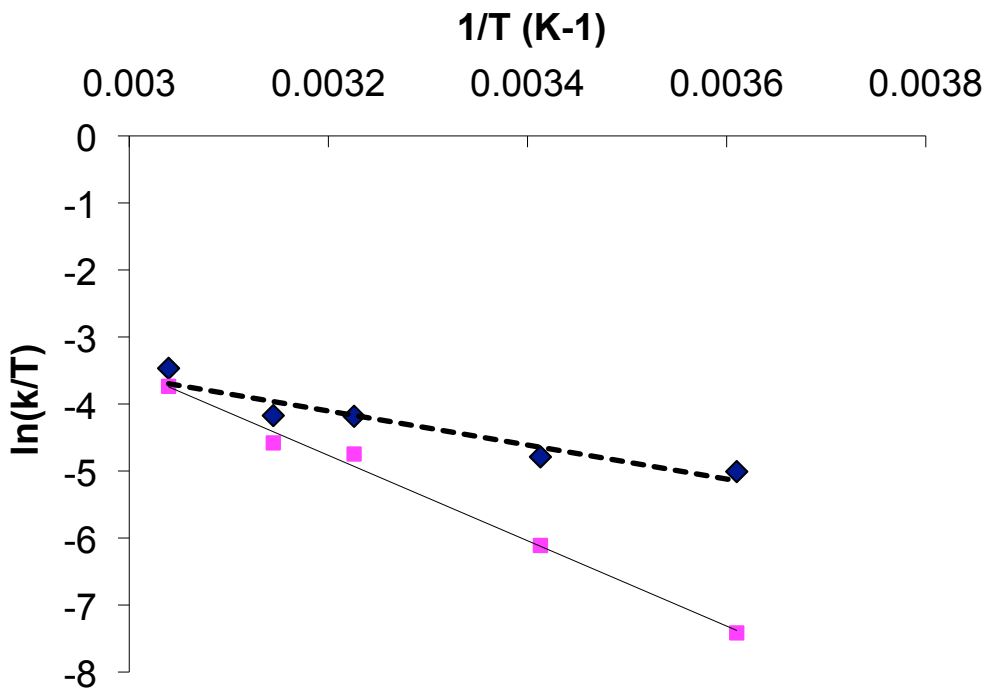


**Figure 4.8 Erying plot of four and six month deg studies shown in blue and pink respectively**

By converting the data to give an Eyring plot as shown in Figure 4.8, and averaging the data, it was possible to estimate the product shelf life at various temperatures. This is shown in Table 4.9 which shows that at room temperature losses of just over 10% can be expected. This is not therefore an ideal formulation as regards long term storage at room temperature (typically up to 2 years). However it is important to remember that this formulation was never optimised for long – term storage and so if storage was included as a factor in the DoE, then the ideal windows of operation for such a formulation could potentially be obtained.

The Eyring plot in Figure 4.8 also show that the two decay lines fit well to a straight line, which is significant as it demonstrates that protein degradation is only occurring via one chemical mechanism, or at least that the set of chemical mechanisms do not change relative to each other with temperature. Mechanistic changes tend to result in curved or kinked Eyring plots where the two mechanisms have different activation energies. The linear relationship is important as it means that accelerated degradation studies at higher temperatures, accurately reflect the nature of the much slower degradation at lower temperatures. Therefore, the accelerated studies are a good mimic of long term degradation.

| Temperature | % loss per month | % loss per year |
|---|---|---|
| *-20* | 0.046 | 0.547 |
| *4* | 0.322 | 3.778 |
| *20* | 0.984 | 11.157 |
| *37* | 2.821 | 29.06 |

**Table 4.9 Estimated shelf life of 100 ng/ml G-CSF lyophilized in Tween-20 and HSA at pH 7 and various storage temperatures**

## 4.4 Conclusions

Currently the processes by which formulations for lyophilization are chosen are primarily decided via empirical methods. There exists a need for a systematic approach to determining which excipients are most suitable. One approach to date has involved developing a deeper scientific understanding of the interaction between the protein and the excipients as well as an understanding of how the formulation responds to freeze drying (31, 108, 109). However the

information gained from analyzing the protein and its structure will still be insufficient to allow the accurate determination of which cryoprotectants and lyoprotectants will work.

The approach taken here has been to develop a systematic method by which one can quickly identify which excipients have a positive effect and which have a negative or zero effect on preserving biopharmaceuticals with confidence. The use of factorial design also allows the investigator to identify possible interaction effects between excipients. A scientific understanding of the protein molecule is still necessary to identify a set of excipients which are likely to work. However, a factorial designed experiment remains the only way to identify and then optimise a formulation.

The use of microtitre plates has two advantages. The use of ultra scale – down has already been identified as an excellent method of process development investigations using minimal material. The use of automation combined with ultra scale down in areas such as chromatography, filtration and fermentation would require modification or retro fitting of existing equipment to conduct these processes. However, the use of a Tecan or HP robot simply to mix and blend the formulations created via the factorial design software would increase the efficiency of the method immediately. A micro freeze drier, which is a relatively simple piece of kit to manufacture, would increase the viability of this method even further (110). As things stand most scientific establishments have ready access to microtitre plates and robots and the platform technology detailed here could readily be adapted for use in industry immediately.

We have shown through the use of a model protein and an industrially manufactured biopharmaceutical that factorial design coupled with ultra scale down can rapidly identify and optimise formulations for freeze drying to preserve the biopharmaceutical product. The results are easily transferable to manufacturing scale vials. The data produced can be used to develop mathematical models with defined degrees of accuracy vital for validation work. The next chapter will deal with the use of factorial design for development of the lyophilization cycle and will also assess the viability of microtitre plates for such work.

# Chapter 5. Development of a Correlation Model for the Lyophilization Cycle Through the Use of Factorial Design

## 5.1 Introduction

So far this thesis has looked solely at formulation development, with the aim to develop a systematic approach to the creation of viable formulations for the lyophilization of biopharmaceuticals. The aim of such an approach would be to maintain protein structure and activity throughout the freeze-drying process. This Chapter looks at the development of the freeze-drying cycle itself to result in a lyophilized protein cake with a solid appearance and moisture content of below 1%.

While there is no doubt that the cycle parameters and final moisture content impact on the final activity of the protein (111), due to the nature of the investigation outlined herein and the variability of protein assays, preservation of activity was deemed of secondary importance to appearance and moisture content. Indeed it is almost certain that there exists an interaction between formulation and cycle parameters, for example if one freeze-dries mannitol too quickly vial breakage is a common result (112). Using a factorially designed experiment to understand this interaction would be a valuable investigation but it was felt that the initial step towards such an investigation would be to isolate formulation and cycle parameters and investigate each factor independently by assessing the effect of formulation on activity and cycle design on cake quality and moisture content.

From both an operational and regulatory point of view an understanding of what is happening during a process is required. This is particularly true of

lyophilization which is often the final process the biopharmaceutical goes through before it reaches the end user. Furthermore from a process development point of view it is difficult to design a process without knowledge of what is happening during that process. Despite this, the process development of lyophilization is only slightly less empirical than the formulation development (72). The process is generally split into three stages namely freezing, primary drying and secondary drying. The freezing stage can also contain annealing stages whereby ice crystal size is encouraged to grow by holding the frozen solid at temperatures that exceed the glass transition temperature (12), but during the initial stages of process development it is often necessary to simplify as much as possible.

Mathematical models are commonly used in the design of processes in every industry such as bioprocessing, finance or construction. Mathematical modelling of heat transfer to the vial during lyophilization has previously been carried out (77). Furthermore, Boss et al (113) put forward a mathematical solution for modelling the freeze drying process. Their model requires an initial investigation to empirically determine model parameters such as cake density and porosity. One would also probably require a computer programme to calculate optimum cycles based on their model.

While their model has many benefits and is a massive step forward in process development for lyophilization cycles, each formulation has different characteristics in terms of densities, porosities etc. The lyophilization cycle itself will also affect those characteristics. Freezing more rapidly and with no annealing step typically results in a denser cake with lower porosity than a material frozen

slowly. This is due to differences in the size of the ice crystals formed during the freezing step. Vials tend to freeze from the bottom up resulting in the non-uniform morphology of ice crystals and the resulting cake structure (114, 115). These will result in deviations from a model system. Additionally, the existing models cannot predict occasions where the cake collapses. Although it can be generalised that a collapsed cake will often have higher residual moisture content, it does not follow that by a cycle determined by a model to result in a cake with low residual moisture would result in a uniform solid cake structure. For example, a model may suggest a high pressure difference and high temperature to drive the process forward as fast as possible. However, the resulting presence of high levels of protein on the surface of the cake can form a skin which "bursts" under the pressure of the water vapour causing a collapsed cake.

This investigation aimed at developing an approach that was generic in its method and application but that would result in a product specific result every time, in order to determine the optimum cycle parameters to minimize residual moisture content while guaranteeing the formation of a freeze dried cake. The aim of such an approach is to put the controller in a position whereby they understand their system allowing them to demonstrate Quality by Design (QbD). By utilising such knowledge the user is able to state with conviction what is occurring in their process. Furthermore, accidents such as vacuum loss or shelf temperature variations may not necessarily result in the destruction of the product batch within the drier if the operator is able to continue the process utilising the QbD to ensure the resultant product meets the required specifications.

## 5.2 Materials and Methods

PEG (MW 2000), Sodium Pyruvate, Sodium Phosphate (monobasic and dibasic) and Lactate Dehydrogenase (from Lactobacillus leichmanii) were all purchased from Sigma-Aldrich Chemical Co (Poole, UK). NADH was purchased from Calbiochem (Nottingham, UK), lactose from VWR (Leicestershire, UK). LDH (282 U/mg) was purchased as a lyophilized powder and was resuspended for baseline assaying in 100 mM phosphate buffer at pH 7.0 or for lyophilization in 50 mM phosphate buffer at pH 7.0 and diluted to the desired concentration.

### 5.2.1 Determination of residual moisture content

Residual moisture analysis was performed using a coulometric Karl Fischer (Mitsubishi CA-100, A1-Environsciences, Luton, Bedfordshire, UK) on three samples of each run. The coulometer performance was checked before assaying the products using a water standard (Solution P, A1 Environsciences) with a value of 180-210 μg water per 50 μL injection with a CV of <5%.

### 5.2.2 Determination of the glass transition temperature of LDH formulated in phosphate buffer, 0.41% PEG and 1.7% Lactose using modulated differential scanning calorimetry

Modulated Differential Scanning Calorimetry (mDSC) was carried out using a Q2000 DSC (TA Instruments, Crawley, UK). 40 μL of the formulation was pipetted into a DSC pan, crimped closed and placed together with an empty reference pan on the autoloader. The sample was lowered to -90 $^{o}$C at 10 $^{o}$C/min then raised at a rate of 1.5 $^{o}$C/min up to 25 $^{o}$C with modulations at 0.23 $^{o}$C every

60 seconds. Analysis was performed in Universal Analysis (TA Instruments, Crawley, UK)

### 5.2.3 Determination of the Correlation Model for the Lyophilization Cycle for LDH

A central composite face design was used with response surface modelling to develop a mathematical model to identify an optimum cycle design that minimized the residual moisture content while maintaining a good cake structure appearance. To simplify the design no annealing stage was used, ramping rates were fixed at the limits of the machine, parameters such as temperature and pressure were kept constant within each stage and the process was split into a freezing, primary drying and secondary drying stage. As the driving force during primary drying can be expressed as the difference between vapour pressure and chamber pressure (14) the chamber pressure during the primary drying phase is expressed as a percentage of the vapour pressure at that shelf temperature. The factors and their ranges are shown in Table 5.1.

| Name | Units | Low | High |
|---|---|---|---|
| Freezing Temp | C | -40 | -21 |
| Freezing Time | mins | 30 | 240 |
| PD Temp | C | -21 | -11 |
| PD Time | mins | 360 | 720 |
| PD Pressure | % Vp | 10 | 60 |
| SD Temp | C | 0 | 30 |
| SD Time | mins | 0 | 720 |
| SD Pressure | mTorr | 30 | 100 |

**Table 5.1 Factors and ranges investigated to develop a model**

A central composite face design was developed using Design Expert 7 and was used to vary each factor between its maximum, minimum and mid point. A full list of all the combinations used is shown in Table 5.2. Each run involved the lyophilization of 10 vials containing 1 ml of solution containing 0.05 U/ml LDH,

1.7% Lactose and 0.41% PEG in pH7 50 mM Phosphate Buffer. Following lyophilization, all the vials were examined visually to determine the quality of the cake. The cake quality was qualified as simply either good (1) or poor (0). The presence of any vial containing a collapsed cake other than the vial holding the thermocouple resulted in a score of 0. Triplicate vials were then assayed for residual moisture content using Karl Fischer Titration. Following incubation at room temperature for at least two weeks, duplicate vials were assayed using NADH and pyruvate on a plate reader as has already been described in section 3.2.3.

| Run | Freezing Temp | Freezing Time | PD Temp | PD Time | PD Pressure | SD Temp | SD Time | SD Pressure |
|---|---|---|---|---|---|---|---|---|
| | C | mins | C | mins | % Vp | C | mins | mTorr |
| 1 | -21 | 240 | -21 | 360 | 60 | 0 | 0 | 30 |
| 2 | -21 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 3 | -40 | 30 | -11 | 360 | 50 | 0 | 0 | 30 |
| 4 | -21 | 240 | -11 | 720 | 10 | 30 | 720 | 30 |
| 5 | -40 | 240 | -21 | 720 | 10 | 30 | 0 | 100 |
| 6 | -21 | 240 | -21 | 360 | 60 | 30 | 720 | 30 |
| 7 | -30.5 | 135 | -16 | 720 | 35 | 15 | 360 | 65 |
| 8 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 30 |
| 9 | -21 | 30 | -11 | 360 | 10 | 30 | 720 | 30 |
| 10 | -40 | 30 | -21 | 720 | 60 | 0 | 0 | 30 |
| 11 | -21 | 30 | -21 | 720 | 10 | 30 | 720 | 30 |
| 12 | -40 | 240 | -11 | 360 | 50 | 30 | 720 | 30 |
| 13 | -30.5 | 30 | -16 | 540 | 35 | 15 | 360 | 65 |
| 14 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 15 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 100 |
| 16 | -21 | 240 | -21 | 720 | 10 | 0 | 0 | 30 |
| 17 | -30.5 | 135 | -16 | 540 | 35 | 15 | 0 | 65 |
| 18 | -21 | 240 | -21 | 720 | 60 | 0 | 720 | 100 |
| 19 | -40 | 240 | -21 | 360 | 60 | 30 | 720 | 100 |
| 20 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 21 | -21 | 30 | -11 | 720 | 10 | 0 | 720 | 100 |
| 22 | -40 | 30 | -21 | 720 | 10 | 0 | 720 | 100 |
| 23 | -40 | 240 | -11 | 720 | 10 | 0 | 720 | 100 |
| 24 | -21 | 30 | -21 | 360 | 10 | 0 | 0 | 30 |
| 25 | -21 | 240 | -11 | 360 | 10 | 0 | 0 | 30 |
| 26 | -40 | 30 | -21 | 360 | 10 | 30 | 0 | 100 |
| 27 | -21 | 30 | -11 | 720 | 60 | 0 | 0 | 100 |
| 28 | -21 | 240 | -11 | 360 | 50 | 0 | 720 | 100 |
| 29 | -21 | 30 | -21 | 360 | 60 | 30 | 0 | 100 |
| 30 | -40 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 31 | -40 | 240 | -11 | 360 | 10 | 30 | 0 | 100 |
| 32 | -40 | 30 | -11 | 720 | 10 | 0 | 0 | 30 |
| 33 | -21 | 30 | -11 | 720 | 60 | 30 | 720 | 100 |
| 34 | -30.5 | 135 | -16 | 360 | 35 | 15 | 360 | 65 |
| 35 | -30.5 | 135 | -16 | 540 | 35 | 15 | 720 | 65 |
| 36 | -40 | 30 | -21 | 360 | 60 | 30 | 720 | 30 |
| 37 | -21 | 30 | -21 | 360 | 60 | 0 | 720 | 100 |
| 38 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 39 | -40 | 240 | -21 | 360 | 10 | 0 | 720 | 30 |
| 40 | -40 | 30 | -11 | 720 | 50 | 0 | 720 | 30 |
| 41 | -40 | 30 | -11 | 360 | 10 | 0 | 720 | 100 |
| 42 | -40 | 240 | -21 | 360 | 10 | 0 | 0 | 100 |
| 43 | -30.5 | 135 | -11 | 540 | 35 | 15 | 360 | 65 |
| 44 | -21 | 240 | -21 | 360 | 10 | 30 | 720 | 100 |
| 45 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 46 | -30.5 | 135 | -16 | 540 | 60 | 15 | 360 | 65 |
| 47 | -40 | 240 | -21 | 360 | 10 | 30 | 0 | 30 |
| 48 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 49 | -30.5 | 135 | -16 | 540 | 10 | 15 | 360 | 65 |
| 50 | -21 | 30 | -11 | 720 | 10 | 30 | 0 | 100 |
| 51 | -30.5 | 135 | -21 | 540 | 35 | 15 | 360 | 65 |
| 52 | -40 | 240 | -11 | 720 | 50 | 0 | 0 | 30 |
| 53 | -30.5 | 135 | -16 | 540 | 35 | 0 | 360 | 65 |
| 54 | -30.5 | 135 | -16 | 540 | 35 | 30 | 360 | 65 |
| 55 | -21 | 30 | -11 | 720 | 50 | 30 | 0 | 30 |
| 56 | -30.5 | 240 | -16 | 540 | 35 | 15 | 360 | 65 |
| 57 | -30.5 | 135 | -16 | 540 | 35 | 15 | 360 | 65 |
| 58 | -21 | 240 | -11 | 720 | 50 | 30 | 0 | 100 |
| 59 | -40 | 240 | -21 | 720 | 60 | 30 | 720 | 30 |
| 60 | -40 | 30 | -11 | 720 | 50 | 30 | 0 | 100 |

**Table 5.2 Lyophilization cycles used to determine the empirical model**

### 5.2.4 Residual Moisture Analysis Using Karl-Fischer Titration

Residual moisture analysis was performed using a coulometric Karl Fischer (Mitsubishi CA-100, A1-Environsciences, Luton, Bedfordshire, UK) using Aquamicron catholyte and anolyte reagents (Mitsubishi, Japan) within a Plas Labs (A1 Safetech, Luton, UK) nitrogen dry box maintained at low relative humidity (<300 ppmv water vapour as measured by dew point hygrometer DS2000 – 1, Alpha Moisture Systems, Bradford, UK). Three samples from each run were opened within the dry box, reconstituted with anolyte which was then returned to the coulometer. The coulometer performance was checked before assaying the products using a water standard (Solution P, A1 Environsciences) with a value of 180-210µg water per 50µL injection with a CV of <5%.

### 5.2.5 Validation of the Correlation Model

Having attained a mathematical model for predicting the residual moisture and cake structure of the freeze dried product it was necessary to demonstrate that it correlated well with real life examples. Five different cycles were selected and repeated in triplicate to assess the accuracy of the model. One cycle prediction was outside the limits of the initial investigation to determine how accurate the model was for predictions beyond the limits of the data obtained.

## 5.3 Results and Discussion

### 5.3.1 Determination of the glass transition temperature of LDH formulated in phosphate buffer, 0.41% PEG and 1.7% Lactose

Differential Scanning Calorimetry (DSC) is routinely used to determine the glass transition temperature of formulated materials (116-121). The

thermogram of 0.5 U/ml LDH formulated in 50 mM phosphate buffer, 0.41% (w/w) PEG and 1.7% (w/w) lactose at pH 7.0 obtained by mDSC is shown in Figure 5.1 and demonstrates that an exothermic (crystallization) event occurred at -15 $^{o}$C. To allow for any errors in the monitoring of the shelf temperature and to take into account the heat transfer from the shelf to the vial, -16 $^{o}$C was selected as the significant temperature above which collapse was expected during primary drying.

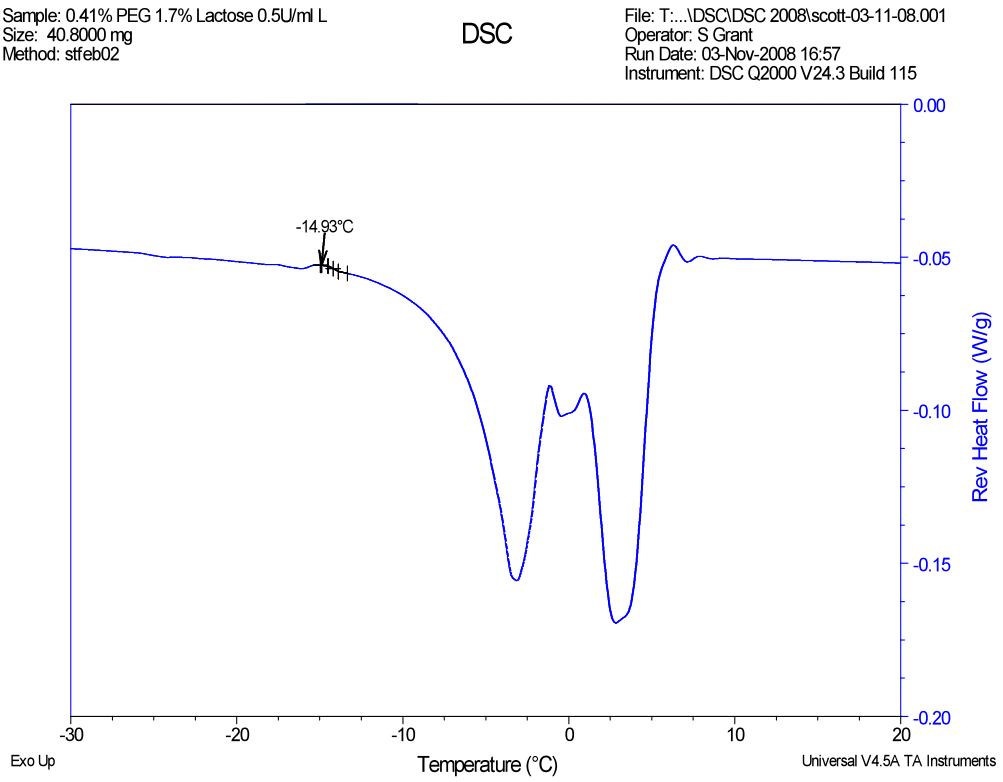


**Figure 5.1 mDSC thermogram of 0.41% PEG, 1.7% Lactose and 0.5 U/ml LDH**

### 5.3.2 Correlation Model for Residual Moisture Content

A typical lyophilization cycle used is shown in Figure 5.2.

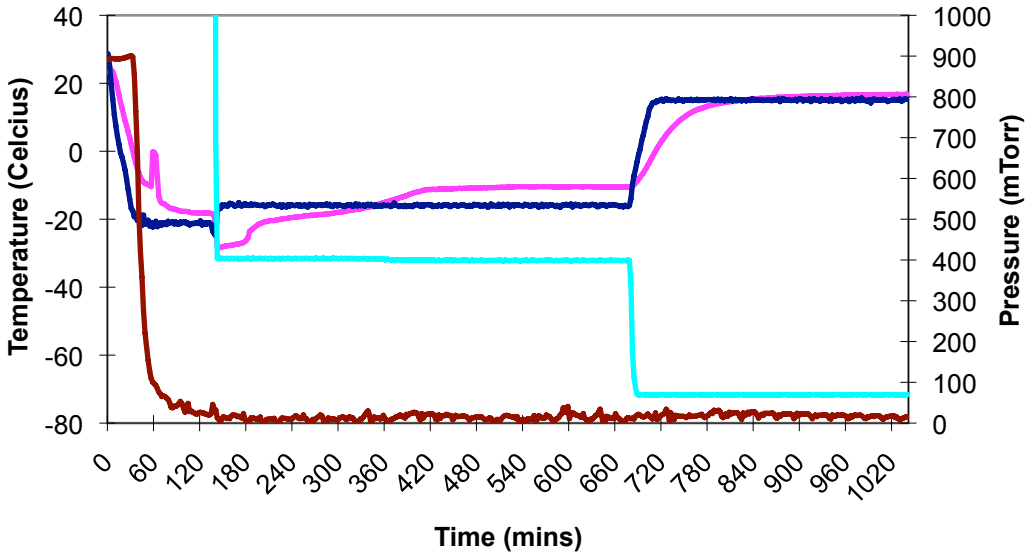


**Figure 5.2 Lyophilization cycle for Run 2. As shown in Table 5.2, Run 2 started with a 240 min freeze at -21 $^{o}$C, then primary drying took place for 360 mins at – 21$^{o}$C and 396 mTorr (60% of the vapour pressure of ice at – 21 $^{o}$C). Secondary drying was conducted for 360 mins at 15 $^{o}$C and a pressure of 65 mTorr. The pink, dark blue, light blue and brown lines represent product temperature, shelf temperature, chamber pressure and condenser temperature respectively**

During primary drying, the water present as ice sublimes to leave a solid with approximately 10% w/w moisture content. This remaining water is released during the secondary drying stage. The presence of bulk water allows the protein product to remain biologically active but also provides potential conditions for the protein to aggregate or unfold. By removing more than 99% of the bulk moisture content the final product is immobilized in the cake structure and less likely to decompose. The residual moisture content can be assessed using Karl Fischer titration. Following freeze drying, three vials were assessed for residual moisture

content using a Karl Fischer titration cell and the results averaged before input and analysis to determine a model. The resultant model is shown in Equation 5.1.

$$MC = \left(5.56 - (3.85\times10^{-3})t_F - (2.17\times10^{-3})t_{PD} + 0.01P_{PD} - 0.02t_{SD} - (1.93\times10^{-3})T_{SD}\right)^2$$

**Equation 5.1 Correlation model for residual moisture content (MC = Moisture Content, F = Freezing, PD = Primary Drying, SD = Secondary Drying, t = time (mins), T = Temp (oC), P = Pressure (%Vp at that temperature for PD and mTorr for SD)**

What is immediately apparent is that out of the eight factors from Table 5.1 only five are shown to have a significant impact on the residual moisture content. It would appear that as long as the freezing temperature is below the glass transition point of the material the actual temperature is insignificant. In an industrial process a low freezing temperature is vital to avoid the occurrence of supercooling, which by way of example, demonstrates the importance of combining engineering knowledge as well as a good model.

By inspection of the equation it is clear that the driving forces behind the rate of freeze drying are the chamber pressure during primary drying and the length of the secondary drying phase. While the shelf temperature during primary drying has been ignored in the model, it is important to bear in mind that the chamber pressure during primary drying is expressed as a percentage of the vapour pressure of ice at the selected shelf temperature. As such the shelf temperature has already been incorporated into the model as the primary drying phase chamber pressure is a derived factor rather than a base factor. The shelf temperature during primary drying has been shown to be irrelevant in determining the rate of freeze drying on condition that the product temperature does not exceed

the collapse temperature (78). The product temperature is a far more important factor but no method for controlling the product temperature has been successful to date.

The pressure difference between the vapour pressure on top of the ice front and the chamber pressure has previously been shown extensively to influence the rate of freeze drying (14, 78, 122). In fact the fundamental modelling of freeze drying often uses the relationship shown in Equation 5.2. A high chamber pressure results in a small pressure difference and a lower rate of sublimation which results in a higher moisture content. As the term PDP (Primary Drying Pressure) in Equation 5.1 relates to a percentage of the vapour pressure at that shelf temperature, the term has in fact taken Equation 5.2 into account.

$$\frac{dm}{dt} = \frac{P_o - P_c}{R}$$

**Equation 5.2 Where: dm/dt = rate of sublimation, Po = vapour pressure of ice at that product temperature, Pc = chamber pressure and R = resistance to vapour flow**

The most significant factor in the model, seen in fact to be twice as important as the primary drying pressure, is the length of time of the secondary drying phase. The secondary drying pressure, which was varied between 30 and 100 mTorr, is shown not to have any effect at all. In other words, there is no need to run the secondary drying phase below 100 mTorr as it will make little difference to the drying rate. This is because the rate determining step during

secondary drying has been shown to be the diffusion of water through the solid (37, 122).

Diffusion is the random migration of molecules from a region of high concentration to a region of low concentration and is defined by Brownian Motion. As such it is logical to state that the more time the particles have to migrate and the more energy in the form of heat inputted into the system, the greater the rate of diffusion of water particles. Pressure would not be expected to significantly impact on the rate of diffusion of vapour through the solid. The results from this experiment correlate well with those found by Pikal et al (37) and others.

Pikal et al (37), showed that not only is chamber pressure not a significant factor during secondary drying but also discovered that the rate of drying during the secondary drying phase is initially high but then plateaus out. High shelf temperatures can reduce the height of the plateau as confirmed by Equation 5.1. Therefore if the rate of drying plateaus and cannot be made to increase it is understandable that the length of time of the secondary drying phase is an important factor in minimizing residual moisture content.

It is interesting to note that the length of time for which the product underwent freezing had a significant impact on moisture content. The freezing period was varied between 0.5 hours and 4 hours. It was expected that freezing for 0.5 hour at a temperature below the glass transition temperature of the product would be sufficient to freeze 1ml. Furthermore as freezing is essentially a binary

state (either it is solid or liquid) it was not expected to influence residual moisture (although it may effect appearance as if it doesn't freeze it will collapse). However the model shows that freezing time can have an impact on the residual moisture content. This suggests that something must have been happening within the vial during the freezing stage – such as annealing.

As has been shown previously (52, 225, 122, 123), freezing rate is also important. By allowing the ice crystals time to grow in size one increases the pore sizes in the freeze dried cake when the ice sublimes. A more porous structure allows sublimation to occur at a faster rate resulting in lower final residual moisture content.

Finally the primary drying time is also seen to be a significant factor in reducing the residual moisture. This is logical because 90% of the drying takes place during the primary drying and that takes time. Furthermore there is some overlap between the primary and secondary drying stages and some evaporation and diffusion can still occur at the lower temperatures present during primary drying.

### 5.3.3 Correlation model for cake structure

Although the cake structure has few implications towards activity and quality of the product, manufacturers prefer the finished lyophilized product to have a cake with a solid appearance and little evidence of shrinkage. This is generally assessed by eye by the operator and measured as either good or bad. For the model it was necessary to quantify this assessment and so was done by

awarding either a 1 for a good cake or 0 for poor cake. The assessment was carried out on the batch rather than the individual vial such that if 9 vials had a good appearance and 1 vial had a broken cake, the batch was awarded a 0. This was because the aim of the model was to achieve 100% perfection or as close to perfection as possible. 1 collapsed cake in 10 is 10% which was deemed too high a failure rate. To avoid bias on behalf of the operator, the majority of the samples were also assessed independently by the freeze drying staff at NIBSC.

Technically one could argue that as a collapsed cake contains higher residual moisture content, cake structure is included in the moisture content. However this is not the case as a product could have very low moisture content and still have collapsed due to blow out or through drying too quickly.

Following the assessment of sixty runs the results were processed by Design Expert 7 to produce the correlation model as shown in Equation 5.3.

$$CS = 4.56 + 0.20T_F - (2.76\times10^{-3})_F + 0.01T_{PD} + (5.02\times10^{-3})_{PD} - 0.05P_{PD} - 0.02T_{SD} - (2.28\times10^{-4})_{SD} - (2.52\times10^{-3})P_{SD} + (4.56\times10^{-5})T_F P_{PD} + (4.90\times10^{-5})T_F P_{SD} - (5.83\times10^{-5})T_{PD}\ t_{SD} + (3.42\times10^{-4})P_{PD} T_{SD} - (8.35\times10^{-6})_{SD}\ P_{SD} + (3.04\times10^{-3})T_F{}^2 + (4.14\times10^{-4})P_{PD}{}^2$$

**Equation 5.3 Correlation model for cake structure where: CS = Cake structure, T t and P are Temperature time and pressure respectively, subscripts F, PD and SD are freezing, primary drying and secondary drying phases**

The impact of each factor can be assessed by its coefficient. A high coefficient means that factor has a high impact on cake structure. Except in the cases of freezing and primary drying temperatures, a positive coefficient means the factor increases the likelihood of a good cake structure and a negative one implies a decreased likelihood. The reverse is true for freezing and primary drying temperature as the temperature itself is inputted as a negative value. Therefore we can order the factors in terms of their coefficients to determine the order in which they each influence a good cake structure as shown in Table 5.3. The fact that the freezing temperature and primary drying pressures have a quadratic relationship with the cake structure demonstrates that the optimum value for these factors lies within the experimental parameters.

| Co-Efficient | Factor |
|---|---|
| 3.04E-03 | Freezing Temp^2 |
| 5.02E-04 | PD Time |
| 4.14E-04 | PD Pressure^2 |
| 3.42E-04 | PD Pressure * SD Temp |
| -5.83E-05 | PD Temp * SD Time |
| 4.90E-05 | Freezing Time * SD Pressure |
| 4.56E-05 | Freezing Time * PD Pressure |
| -8.35E-06 | SD Time * SD Pressure |
| -2.28E-04 | SD Time |
| -2.52E-03 | SD Pressure |
| -2.76E-03 | Freezing Time |
| 1.06E-02 | PD Temp |
| -1.60E-02 | SD Temp |
| -5.16E-02 | PD Pressure |
| 2.04E-01 | Freezing Temp |

**Table 5.3 Table of factors ordered by coefficient. The factors that have the highest positive impact on cake structure are listed in descending order**

The freezing temperature is seen to have the greatest impact on cake structure as it is at the top and bottom of Table 5.3. It is necessary to assess the

overall impact of freezing temperature within the system. To do that one can make all the other factors constant and assume freezing temperature is the only variable. The resulting equation is shown in Equation 5.4. The limits for freezing temperature were -21 oC to -40 oC. For any temperature within that range the square of the temperature would be positive while the temperature itself is negative. The coefficient of TF is 0.2 or -0.2 multiplied by the modulus of TF. The coefficient of TF2 is 0.00304 and remains positive. Although TF2 is always one order of magnitude higher than TF, the coefficients imply that 0.20TF is always going to be one order of magnitude *higher* than (3.04x10-3)TF2. As 0.20TF is negative, a high freezing temperature is required to attain a good cake structure.

$$CS = k + 0.20T_F + \left(3.04 \times 10^{-3}\right)T_F^{\,2}$$

**Equation 5.4 Equation showing relationship between cake structure and freezing temperature where CS = Cake Structure, T = Temperature and subscript F = Freezing stage and k is a constant**

The Tg' of the test material as measured by mDSC was -16 $^{o}$C (see section 5.2.2). The freezing temperature range investigated was between -40 $^{o}$C and -21 $^{o}$C with ramp rates of 1 $^{o}$C/min. The samples were held at 5 $^{o}$C for two hours and then the shelf temperature dropped to the set limit. A low shelf temperature would have resulted in faster freezing and consequently smaller ice crystals. Smaller crystals lead to smaller pore sizes which thus imply that the ice sublimation will take place at a slower rate due to an increased resistance to diffusive flow. As such, if the primary drying settings were particularly forceful

(high shelf temperature and low pressure) the high flow of vapour could collapse the cake structure. Therefore, freezing at a higher temperature closer to the Tg' would allow larger ice crystals to form which minimise or prevent this problem.

The other possible cause of cake collapse due to low freezing temperature is the rapidity with which the shelf temperature changed at certain settings. The primary drying temperature range was between -21 $^{o}$C and -11 $^{o}$C (five degrees plus or minus the Tg') which meant that at a TF at the higher end of its range, the difference in shelf temperature between freezing and primary drying was 0 $^{o}$C to 10 $^{o}$C, whereas at the lower end of the freezing range the difference was a minimum of 19 $^{o}$C and a maximum of 29 $^{o}$C. Therefore, in an extreme case where the freezing temperature was -40 $^{o}$C and the primary drying temperature was -11 $^{o}$C, the smaller ice crystals caused by rapid freezing would result in a low sublimation rate which may be insufficient to cool the product and offset the higher than Tg' shelf temperature resulting in cake collapse.

Similarly one can observe that a long primary drying phase decreases the likelihood of collapse by ensuring complete sublimation of the free water in the ice phase thereby raising the Tg of the solid cake preventing collapse when the shelf temperature is raised to secondary drying operating temperatures. It can therefore be concluded therefore that an ideal cycle should avoid drastic shifts in shelf temperature and allow longer slower freezing or an annealing stage to maximize crystal size and minimize primary drying time.

It is not possible to assess the individual impact of the remaining factors due to the interactions occurring between them. For example the PD_Pressure*SD_Temp is shown in Table 5.3 to have a high positive impact on cake structure. However using the prediction tool within DesignExpert 7 to calculate predictive results using the model equation, the optimum occurs when the secondary drying temperature is 0 $^{o}$C. This is because SD_temp also impacts the cake structure on its own and has a high negative coefficient. When SD_Temp = 0 this cancels out the negative coefficient resulting in a higher cake structure number.

### 5.3.4 Validation of correlation models

It was necessary to demonstrate that the model equations are capable of accurate predictions. It was further necessary to understand how accurate these predictions are. Five validation experiments were carried out, four using operating parameters within the ranges used for developing the model and one using operating parameters outside the model to observe how true the model is outside the set ranges. Each experiment was carried out in triplicate and the resultant observed vs predicted graph is shown in Figure 5.3. The model also accurately predicted cake structure 100% of the time. The observed results correlate almost exactly with the predicted results and one can therefore rely on the models for designing a process with confidence. The point 10%/3% (predicted/observed) was the one anomaly during the validation investigation. Here the model was set to predict high moisture content with an acceptable cake structure which was pushing the limits of the model as high residual moisture content generally results in a collapsed cake. This experiment did not accurately

predict the result which suggests that the model may not be dependable when pushed to its limits to produce results that are scientifically unlikely to succeed. This demonstrates the fact that although DoE is a valuable tool, it cannot and must not replace scientific thought and process during an investigation.

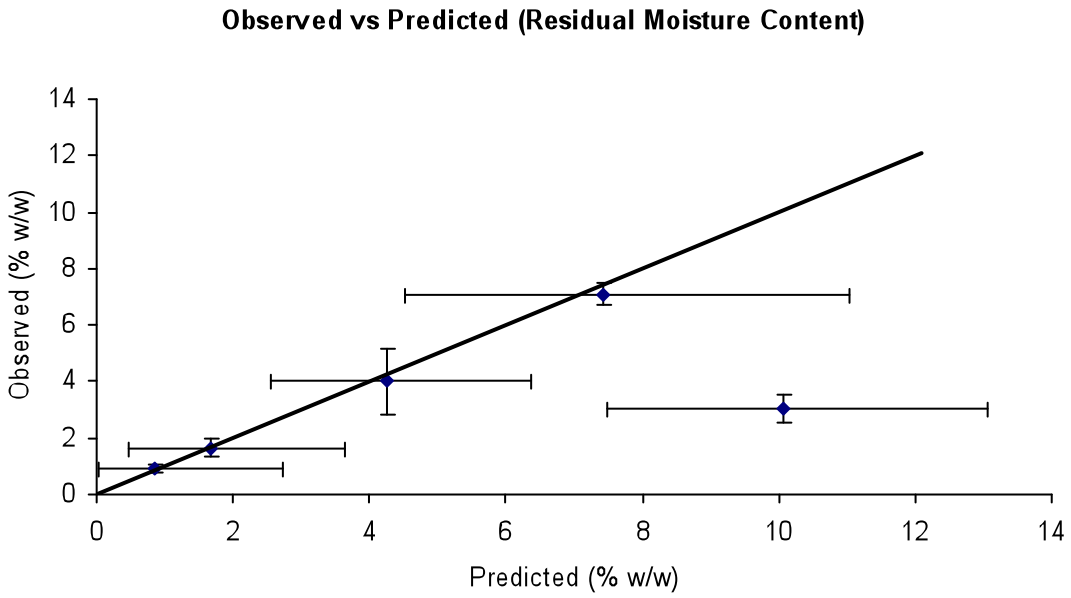


**Figure 5.3 Oberved vs Predicted plot for residual moisture content. Predicted values are shown with corresponding 95% confidence intervals and Observed points are shown with standard errors**

### 5.4 Conclusions

In industrial processes as has often been said time is money. The freeze-drying process itself is a costly process in terms of the energy consumption in creating the low temperatures and low pressures, the capital cost of equipment and the potential losses as the 99.99% pure product is loaded into one machine where failure could mean the loss of millions of pounds of finished product. It is therefore imperative to keep costs down and the process robust.

The results of this chapter demonstrate the ability of DoE to optimize and understand the lyophilization process. There are other factors to be taken into account most notably that of scale. This investigation was carried out in 5 ml vials but only involved 10 vials per run. Although industrially the process would still be carried out in the same vials the process would be scaled out to several thousand vials. Therefore although the models can identify a cycle that would rapidly dry the product, the operator has to take into account the limitations of the equipment and potential for choked flower at the limits of the condenser (15).

DoE is a powerful tool that, as demonstrated in this investigation, can be used to pinpoint optimum process cycles that minimize drying times while ensuring a required quality of product is obtained. However, the user must be wary of the impact of the selected ranges and must analyze the results carefully to avoid reaching incorrect conclusions. There definitely exists an interaction between formulation and the process cycle, where different excipients respond differently to the same conditions. This investigation and the one in the previous chapters allow the user to design a further set of experiments to investigate the optimum formulation under the optimum conditions, possibly involving annealing to perfect a process which results in a biologically active dry product with an acceptable appearance.

# Chapter 6. Use of DoE Derived Models in Process Validation

## 6.1 Introduction

Although it may appear repetitious, as the experiment detailed within has already been detailed in the previous chapter, this chapter looks at the work from a different perspective. It is a requirement of an EngD thesis to demonstrate an understanding of process validation. It has previously been stated that DoE lends itself well to process validation and this chapter highlights an example procedure of how DoE can be applied in such a fashion.

Before any therapeutic is allowed into the market it has to be approved by a relevant organisation. Examples of such organisations include the Food and Drug Administration (FDA) in the USA, the European Medicines Evaluation Agency (EMEA) in Europe and the Medicines and Healthcare Regulatory Agency (MHRA) in the UK. While the approach and minutiae of these agencies may differ slightly in certain areas, the regulatory body will grant approval on condition that the product is safe for administration. In order to demonstrate that ones product meets this requirement, it is necessary to show a thorough understanding of both the therapeutic agent and the process by which it is made.

The safety and efficacy of the product itself is demonstrated through clinical trials. The process must be shown to be sufficiently robust that one can show a consistent quality of product is being manufactured. This is done by validating the entire process from start to finish. The definition of process validation according to the FDA website is:

"Establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes."

In the past this was taken to mean that the engineer had to fix the process and no variation from the approved process was acceptable. Recently however, the idea of process validation has been focussed more on the end product and on a better *understanding* of the process with an aim to ensure a consistent product. In other words, if the process does undergo either deliberate or accidental variation, as long as the operator understands the effect of these variations and can demonstrate that according to the *documented evidence* such a change should not result in the product not meeting specifications, the batch is acceptable for distribution. This is the principle behind *Quality by Design* (QbD).

In some ways process validation is a documented sensitivity analysis, the operator decides on predetermined limits of acceptability for the product and then demonstrates how much variation can take place before the product is unacceptable.

It is a requirement of an EngD thesis that a chapter be devoted to process validation. With this in mind the purpose of this investigation was to validate the process cycle model developed in Chapter 5 to assess its suitability, sensitivity, reliability and limitations.

## 6.2 Materials and Methods

The methods and materials used have already been described in section 5.2.5.

## 6.3 Results and Discussion

Chapter 5 details the development of a product specific model to allow the user to predict cake structure and residual moisture content of a freeze dried product depending on the freeze drying cycle used. Following development of such a model the user would have to validate the model and confirm its accuracy. This can be done by inputting various process parameters into the model to get a prediction and then carry out the modelled cycle to get actual data. These results can be shown in a plot of predicted vs actual moisture content remaining in 5 ml vials after freeze-drying, as shown in Figure 5.3. The first important detail to note is the standard error bars for the observed points. These points are the results of three identical runs and their size indicates the level of consistency the process achieves. In general, industrial lyophilization processes aim to achieve less than 2% residual moisture content (102). The first point in Figure 5.3 shows that not only is this possible but even with the standard error the residual moisture is still below 2% thus demonstrating that the model is capable of predicting final moisture contents to an acceptable standard for validation.

The second point worthy of note is the fact that four out of the five data points lie on the predicted vs observed line. This meant that for 80% of the time, the model was nearly 100% accurate, or rather for any prediction below 8%, the predictions were always correct. Both of these results are important. If a

malfunction or process variability occurs during freeze-dried material production such that the vacuum fails, or secondary drying temperatures are not reached, then the operator could input the actual values for the lyophilization cycle parameters into the model and find out the current moisture content in the vial with a high degree of accuracy *provided the model does not predict a moisture content above 8%.* Were it to do so, then the model cannot be relied on as the actual moisture content could potentially be wholly incorrect. If the prediction gives moisture content as less than 8% then assuming the process is still ongoing, one can work out what further steps are necessary to result in a product that meets the required specifications. While this particular model is only looking at one outcome, moisture content, it could be expanded to include all outcomes specified by the company.

This method of modelling using DoE can therefore be used in a quality by design (QbD) process which, through real-time modelling, has the potential to achieve a form of process analytical technology (PAT).

## 6.4 Conclusion

As process optimisation moves towards quality by design, methods that allow for a fully demonstrable understanding of the process will become more common. This investigation looked at how a statistically designed experiment resulted in a system specific process model that could be used to validate the process and demonstrate quality by design. The results showed the model to be 100% accurate within its limitations but those limitations do not negate the validity of the model as long as the user understands where those limitations lie.

Based on the derived model the user is able to validate the process, monitor the process, conduct sensitivity analysis and trouble shooting.

# Chapter 7. Conclusion

## 7.1 Summary

In Chapter 1 the current state of the art regarding the lyophilization of biopharmaceuticals was discussed. Also, the growing requirement for lyophilization as a method of long term storage was discussed and the current methods used in process development were described. The conclusions of Chapter 1 identified the need for a high-throughput systematic and automatable process for screening multiple variables, and then optimising a lyophilization process and formulation.

High-throughput automation invariably means the use of the microtitre plate format. Robotic liquid-handling systems that work with these plates have been around for a number of years and are becoming more common pieces of equipment in nearly all laboratories. Furthermore, their extension for use in other unit-operation designs has been well documented. In Chapter 2 the the use of microwell plates for lyophilization was characterized and the following conclusions were drawn:

- It is possible to freeze dry formulations in microwells. However, the edges of the microtitre plate must be trimmed to allow good thermal contact with the freeze-dryer shelf.

- There is a linear relationship between the area of the ice-wall interface and the volume of ice that influences the rate of sublimation during primary

drying. Therefore, by identifying the rate of sublimation in a microwell, it is possible to predict the rate of sublimation in a vial.

Having shown that microtitre plates can be used in lyophilization design, the next stage was to develop a systematic approach to freeze-drying biopharmaceuticals. To tackle this, the lyophilization process design was split into two parts: formulation and process. As shall be mentioned in more detail later in this Chapter, there is definitely a link between the process cycle and the formulation. However, an understanding of each of these factors must be first established before looking at how they influence each other.

Lyophilization formulations are generally worked out empirically based on experience and any scientific data available. Quantities and types of excipients are picked semi-randomly and a hit and miss approach is used to work out the optimum formulation. Chapter 3 and Chapter 4 investigated the use of factorially-designed experiments combined with microtitre plates to develop a high-throughput platform technology to identify optimum formulations for preserving biological activity during lyophilization. The key conclusions from these investigations were as follows:

- A fractional factorial-screening design followed by a central-composite optimisation design will identify key factors and display graphically the windows of operation within those factors.

- The window of operation is valid both in microwells and in 5ml vials.

- The models produced allow the operator to validate the formulation and assess the impact of variations in concentrations.

- The number of excipients screened, and the ability to use microtitre plates, means this method can be defined as a high-throughput technique.

Having dealt with formulation development it was necessary to look at cycle design. It can be argued that although cycle design has an effect on preserving biological activity the dominant factor in preserving activity through the cycle is the formulation. Similarly it can be argued that the dominant factor in reducing residual moisture content in the final dried product and presenting an acceptable cake structure is the cycle design. Therefore, the cycle was assessed in terms of cake structure and residual moisture content. It was not possible to measure residual moisture content in microwells due to various limitations already discussed, and so the investigation in Chapter 5 was carried out in 5 ml vials. This limitation does not mean that the procedure outlined in Chapter 5 does not qualify as a high-throughput method as each run was carried out using only ten vials, and theoretically could have been carried out with less. Automated filling machines for vials do exist all the user requires are several small freeze dryers.

Modelling of lyophilization cycles is a challenge as parameters for factors such as cake porosity often have to be assumed. Furthermore, such factors are often influenced by the cycle design. For example, a rapid freeze can lead to low porosity, whereas an annealing step can introduce larger ice crystals and a higher cake porosity. The approach taken in Chapter 5 was to avoid a generic model and develop a system specific model. The advantage of such a model is that it allows

the operator to demonstrate a detailed knowledge of the system and lends itself well to validation. The conclusions drawn were as follows:

- A factorial design investigation will result in a valid model for a given system that can be relied upon for robust cycle design.
- The model can be used for process validation to demonstrate a good level of quality by design.
- The key factors identified by the model correlated well with previous work discussed in the literature.

### 7.2 Implications

If lyophilization formulation engineers adopted the strategy proposed herein and combined it with a liquid handling system, the number of excipients and buffer types that could be screened is limited only by the resultant assay or protein characterization technique. Methods for high-throughput protein characterization are being developed and the use of one of these methods could allow literally thousands of formulations to be screened. In terms of pure formulation engineering one would only screen excipients and buffers that are likely to work, and that are already used in approved biopharmaceuticals or that are generally recognised as safe (GRAS). However, for investigative research it would be valuable to screen many formulations for several protein products as it is possible that patterns may emerge that would allow more accurate predictions in the future while enhancing our understanding of the interactions that take place between excipients and protein during lyophilization and subsequent storage. Although traditional methods could do the same in terms of single factor effects,

the interaction effects that can be identified only through factorial design have to date not been investigated fully. An understanding for example of why too much PEG and lactose result in a damaged protein is important for furthering our understanding of protein interactions. The data gathered from large screens of different proteins can be collated into a library which may reveal how different classes of proteins respond to different excipients. In short, the potential value is huge.

In the immediate term the value of this platform technology is significant. Although hit-and-miss approaches can be successful and make use of experience (which is a valuable tool also), and available scientific data, the ability to pass this knowledge on to new formulation scientists is limited. The high-throughput approaches described in this thesis can be adapted by anyone with a reasonable understanding of the process and protein chemistry. It can be applied immediately as there is no need for special equipment except perhaps an electric saw to trim the edges of the vials. If a formulation exists, this method will find it.

In terms of cycle design it may not be immediately apparent what the value of conducting sixty trial runs adds to using standard cycle design engineering. Lyophilization cycles are no longer 5-7 days long, and although they are probably still over engineered, this is probably by hours rather than days. However, although a cycle can currently be designed, the user will not know at any one point how dry the product is. The user will not know what the effect a variation has on the process which means the process is set in stone and cannot be adjusted ever. Even the use of FTS SMART Freeze Dryer$^{TM}$ technology will only

tell the user the best cycle to use, it will not provide a model that allows for variations. For example, if a production process is moved to a different facility with a different dryer that might have a different temperature variation or chamber pressure, the user cannot know the impact this has on the process and has to start validation from square one. Using the method detailed in Chapter 5 the user can make adjustments and cope with variations with confidence in the outcome of these changes.

In both formulation and cycle design the approach investigated throughout this thesis allows the user to make the statement "the formulation/process selected was selected on the basis of…" as oppose to "the formulation/process selected was chosen because it works," which as regulatory bodies move more towards quality by design approaches and encourage operators to be familiar with their processes, is a significant contribution to the final stages of process design.

### 7.3 Further work

Chapter 5 looked at basic cycle design to obtain a fundamental model thus demonstrating the proof of concept. However, several important factors were kept constant. Shelf ramp rates can make a difference to the final product as too fast a ramp rate for example can result in product collapse (72, 12, 124). Annealing was not investigated at all. Having identified a region of optimum operability it would be beneficial to conduct a further investigation looking at the impact of these factors.

Chapter 5 was carried out in 5 ml vials despite work in Chapter 2 which showed a linear relationship existed between vessels of different dimensions. The reason for this was that Chapter 5 was looking at the impact of cycle design on residual moisture content which could not be measured in microwells. Thermogravimetric analysis involves the heating of small samples of material and measures the mass lost. The mass lost is due to the residual moisture evaporating. A gas chromatography instrument identifies the released vapours and identifies the point at which $CO_2$ is evolved as well as $H_2O$. This is the point at which the protein is decomposing and the moisture evolved is due to thermal decomposition rather than residual moisture. The device can then work out the percentage moisture content in the sample. As the samples are prepared in a nitrogen dry box and are very small, it is possible to scrape samples from the microwells into the pans and assess moisture content in microwells. This would allow full characterization of lyophilization in microwells and enable process engineers to predict full scale lyophilization cycles from microwell experiments. As microwells hold far smaller quantities of material, several cycles could be investigated in the time taken to investigate one cycle at vial scale. This would alleviate the issue with factorial designed experiments in terms of the number of runs required and the time needed to run them.

Following such characterization it would then be possible to combine cycle and formulation design. There is a definite link between formulation and drying cycle, literature already exists regarding mannitol for example and the rate at which it must be frozen to prevent vial breakage. The rate of freezing will to a degree depend on the formulation as will the morphology of the ice and the

existence of crystalline or amorphous regions. The maximum product temperature before product collapse during primary drying is dependant on the $T_g$' and a similar argument can be made for the secondary drying temperature which was shown in Chapter 5 to be a key factor. The $T_g$' is dependant on the formulation. Similarly this thesis has split up the moisture content, cake structure and biological activity retention and grouped them into either formulation dependant or cycle dependant. In truth the cycle design will impact on final biological activity, large ice crystals may crush the protein, a harsh drying stage could dehydrate the protein shell and damage it. This can be prevented with the correct formulation though, so we see a definite link and potential interaction between cycle design, formulation and biological activity. A formulation with a high solid content will take longer to dry and may not be able to achieve <1% moisture content.

There is also the long term stability factor which was touched upon in Chapter 4 where the optimum formulation for preserving G-CSF activity through lyophilization did not successfully preserve activity in long term storage at room temperature. Long term stability is dependant both on the formulation and residual moisture content. Long term stability is arguably the most important factor as the whole purpose of lyophilization in the first place is to provide a long term storage solution for unstable biopharmaceuticals. Therefore, while retaining 100% of the biological activity through the process is a good start, it is not the final solution to the problem the engineer is trying to solve.

It can therefore be said that there is definite value in conducting a combined study of formulation and cycle design combined preferably in microwells to allow a high throughput study. Perhaps one way to conduct such a study would be to do a preliminary screen on formulation and cycle design separately to eliminate factors that are totally insignificant. This would reduce the number of factors to investigate by a significant amount and make the study more manageable.

The design of a liquid handling system with a built in bank of freeze dryers would be the result of such work. The user would be able to input the factors he/she wants to investigate and walk away while the robot and software conduct a full investigation and present an optimum set of results together with sufficient data for quality by design validation.

According to Genetic Engineering and Biotechnology News (125) 25% of the products currently in the biopharmaceutical pipeline will require lyophilization and this number is set to increase to 40% over the next five years. There is constant and growing pressure on the biopharmaceutical industry to provide therapeutics at lower costs to the patient which in turn means pressure on process engineers to quickly and efficiently design economically efficient processes with a proven efficacy and safety. The work conducted in this thesis would allow formulation scientists to conduct final stage process engineering in such a manner to provide value both to the industry and the final consumer

.

# Chapter 8. Appendix I

This section details briefly the investigations that were undertaken but failed to product a positive result

## 8.12 The use of electrical conductance to measure primary drying rates

A miniature probe was developed to measure the electrical conductance of a sample in a microwell. The probe was made of platinum and the dimensions were:

0.26mm thick

1.8mm wide

9.3mm available to fit in microwell (due to spacer)

2.4mm apart

The probe was connected via a lead through to an ultra sensitive conductance meter

The solution was 5% by weight lactose and the sample size was 200 μL

The microwells were Sarstedt 96-well flat bottomed plates

The shelf temperature is set to -35$^{o}$C and the tray is frozen in situ for at least an hour

The vacuum is non adjustable and is left to give as hard a vacuum as possible

Initially a Fluke MegaOhmeter was used which utilized 2000 volts to generate an output. The results from these experiments are shown in Figure 8.1.

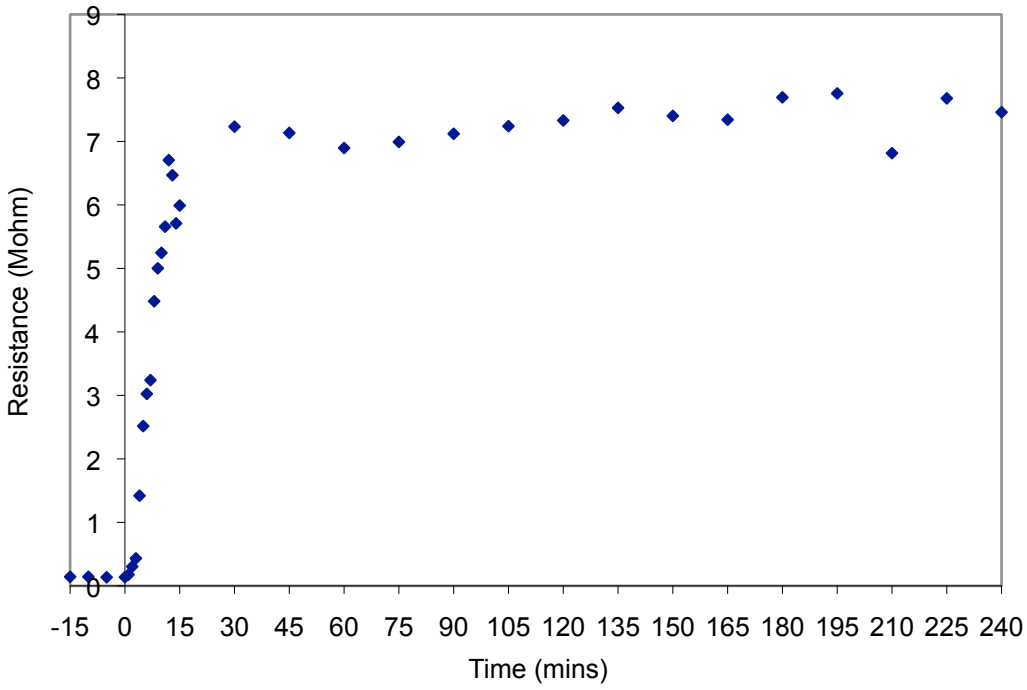


**Figure 8.1 Resistance of a 5% lactose sample during freeze drying with an initial 15 min equilibriation time**

However, the presence of such a large voltage meant that arcing occurred between the probes and a purple parabolic glow could be observed when the system was placed under vacuum.

An ultra sensitive conductance meter was used instead. Due to the sensitivity of the instrument, shielding was necessary to prevent interference. Initially the meter was placed in the drying chamber but the due to the battery freezing a lead through was built and co – axial cabling used to ensure a the probe was adequately shielded. The results from this experiment are shown in Figure 8.2.

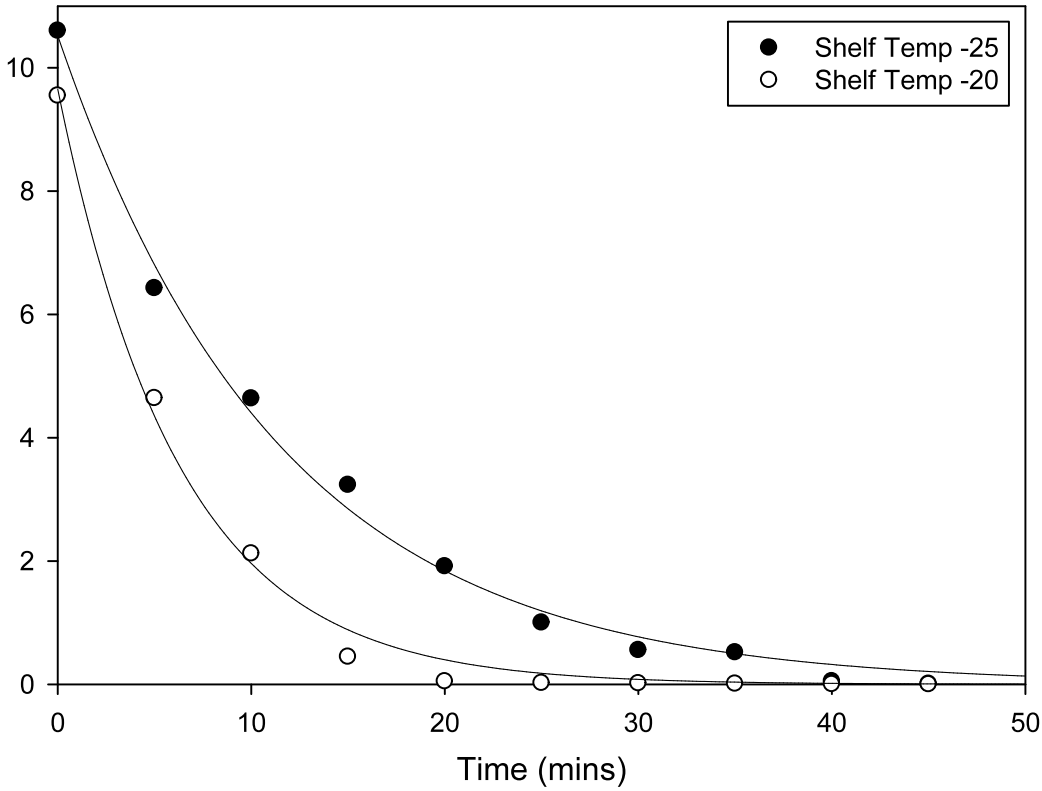


**Figure 8.2 Conductance of sample during primary drying at two different shelf temperatures**

While initial results looked promising, the same trends were observed over time at atmospheric pressure. Obtaining reproducibility was difficult as shown in Figure 8.3.

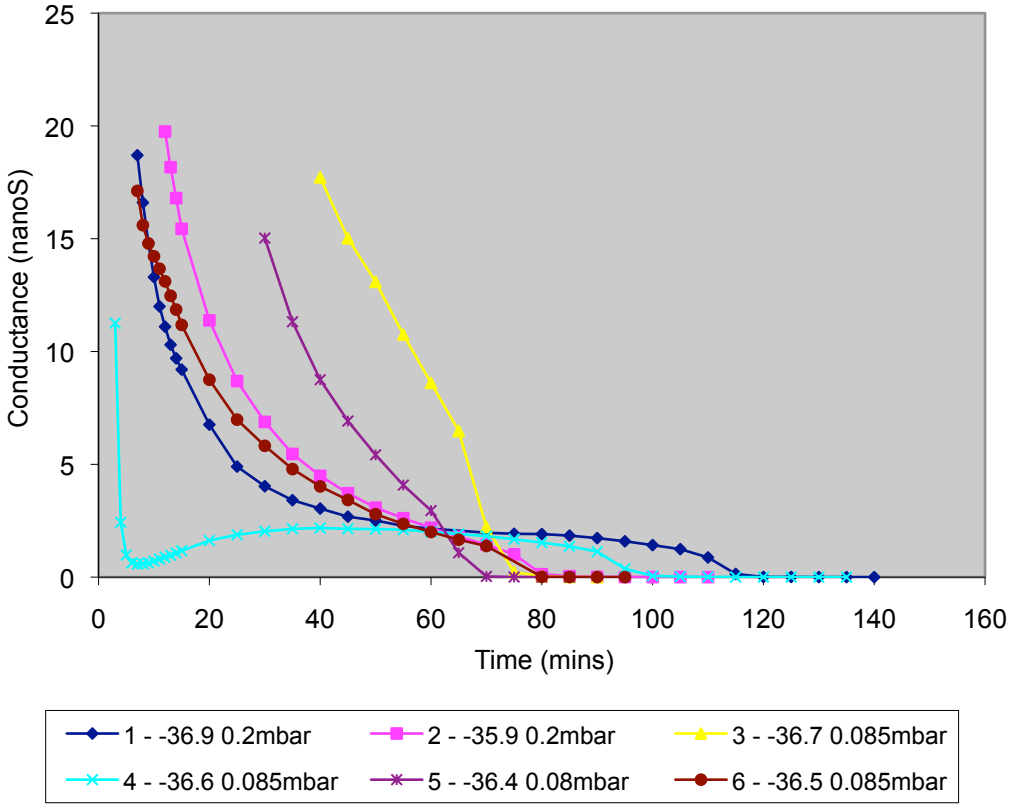


**Figure 8.3 Repeat experiments measuring the conductance of 5% lactose during freeze drying**

It was then observed that the conductance of the material drifted towards zero regardless of the pressure in the chamber. Therefore this line of investigation was brought to a halt.

### 8.13 The use of an infrared probe to measure the primary drying rate

Infra red is readily absorbed by water and the Tunable Diode Laser Absorption Spectroscopy (TDLAS) system which measures the flow of water vapour in the tube between the drying chamber and condenser is an example of the use of infra red in freeze drying (147). This experiment aimed to develop a miniature probe that would sit on top of a vial or microwell and measure the rate of water flowing out of the vessel through the use of an infra red diode and receptor. However no fluctuation in the signal was observed during freeze drying suggesting the vapour flow was too small to be detectable by such a device.

### 8.14 The use of an Near Infra Red Spectroscopy (NIRS) probe to measure primary drying rates in microwells

An NIRS probe measured the reflectance of a sample in a microwell before and after freeze drying. However there was no discernable difference in the traces produced from such a scan.

# Chapter 9. Appendix II

This section contains a selection of the raw data used for different experiments conducted for this thesis.

| 1 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 95 | 125 | 115 | 110 | 125 | 120 | 120 | 115 | 120 | 100 | 95 | 95 |
| B | 115 | 135 | 135 | 135 | 125 | 135 | 130 | 135 | 130 | 120 | 125 | 110 |
| C | 115 | 135 | 145 | 135 | 135 | 135 | 135 | 130 | 135 | 135 | 130 | 110 |
| D | 120 | 135 | 130 | 140 | 135 | 135 | 135 | 130 | 135 | 130 | 120 | 110 |
| E | 125 | 135 | 130 | 135 | 125 | 140 | 135 | 135 | 140 | 135 | 130 | 105 |
| F | 125 | 130 | 140 | 135 | 135 | 140 | 135 | 130 | 120 | 115 | 130 | 110 |
| G | 115 | 130 | 130 | 135 | 135 | 130 | 135 | 130 | 130 | 125 | 125 | 110 |
| H | 105 | 110 | 110 | 105 | 120 | 115 | 110 | 115 | 115 | 110 | 95 | 90 |

| 2 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 105 | 120 | 125 | 120 | 130 | 130 | 125 | 120 | 120 | 115 | 120 | 105 |
| B | 115 | 140 | 140 | 145 | 140 | 140 | 140 | 135 | 140 | 135 | 120 | 115 |
| C | 125 | 140 | 140 | 145 | 145 | 140 | 140 | 145 | 140 | 140 | 130 | 115 |
| D | 125 | 140 | 145 | 145 | 145 | 145 | 145 | 145 | 145 | 145 | 140 | 120 |
| E | 125 | 140 | 145 | 145 | 145 | 145 | 145 | 145 | 145 | 140 | 140 | 125 |
| F | 120 | 140 | 140 | 135 | 145 | 145 | 140 | 145 | 140 | 130 | 140 | 120 |
| G | 120 | 130 | 140 | 145 | 140 | 140 | 140 | 140 | 140 | 135 | 135 | 115 |
| H | 100 | 110 | 115 | 120 | 125 | 120 | 120 | 115 | 120 | 115 | 110 | 100 |

| Average | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 100 | 123 | 120 | 115 | 128 | 125 | 123 | 118 | 120 | 108 | 108 | 100 |
| B | 115 | 138 | 138 | 140 | 133 | 138 | 135 | 135 | 135 | 128 | 123 | 113 |
| C | 120 | 138 | 143 | 140 | 140 | 138 | 138 | 138 | 138 | 138 | 130 | 113 |
| D | 123 | 138 | 138 | 143 | 140 | 140 | 140 | 138 | 140 | 138 | 130 | 115 |
| E | 125 | 138 | 138 | 140 | 135 | 143 | 140 | 140 | 143 | 138 | 135 | 115 |
| F | 123 | 135 | 140 | 135 | 140 | 143 | 138 | 138 | 130 | 123 | 135 | 115 |
| G | 118 | 130 | 135 | 140 | 138 | 135 | 138 | 135 | 135 | 130 | 130 | 113 |
| H | 103 | 110 | 113 | 113 | 123 | 118 | 115 | 115 | 118 | 113 | 103 | 95 |

**Table 9.1 Results from microtitre plate uniformity experiment used for Figure 3.1. Each well was filled with 200 µL water and partially freeze dried. The remaining volume of water was measured using a Hamilton syringe. This experiment was conducted in duplicate.**

| Time (mins) | Run Number | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| 0 | 2.5683 | 2.537667 | 2.617083 | 2.625783 | 2.567733 | 2.57525 | 2.562483 | 2.5563 | 2.56575 | 2.541717 | 2.599683 | 2.495617 | 2.558017 | 2.62095 | 2.654167 | 2.60345 | 2.495783 | 2.640817 | 2.633867 |
| 0.5 | 2.406017 | 2.344933 | 2.4374 | 2.440917 | 2.376217 | 2.424867 | 2.3833 | 2.3798 | 2.40645 | 2.38415 | 2.438183 | 2.331733 | 2.3869 | 2.442633 | 2.440017 | 2.391233 | 2.344283 | 2.455 | 2.433133 |
| 1 | 2.26005 | 2.179533 | 2.287767 | 2.271183 | 2.210083 | 2.2705 | 2.251433 | 2.23195 | 2.2649 | 2.2252 | 2.285583 | 2.149233 | 2.238583 | 2.2735 | 2.263567 | 2.2271 | 2.170483 | 2.313017 | 2.273433 |
| 1.5 | 2.122667 | 2.023833 | 2.138417 | 2.108333 | 2.052633 | 2.132483 | 2.123567 | 2.09405 | 2.136283 | 2.073033 | 2.147317 | 1.978233 | 2.1013 | 2.109467 | 2.093533 | 2.063417 | 1.994367 | 2.182683 | 2.122683 |
| 2 | 1.994033 | 1.873383 | 1.995183 | 1.952383 | 1.901967 | 1.9991 | 2.002033 | 1.967233 | 2.013017 | 1.934417 | 2.016117 | 1.814917 | 1.9721 | 1.95185 | 1.932033 | 1.9072 | 1.831783 | 2.05695 | 1.979717 |
| 2.5 | 1.870833 | 1.731217 | 1.859183 | 1.804217 | 1.7597 | 1.87305 | 1.887033 | 1.846533 | 1.894933 | 1.801467 | 1.8902 | 1.6608 | 1.849933 | 1.803017 | 1.778817 | 1.75895 | 1.678583 | 1.937167 | 1.845483 |
| 3 | 1.752567 | 1.598417 | 1.7339 | 1.664867 | 1.626167 | 1.754233 | 1.779283 | 1.731817 | 1.785 | 1.677 | 1.769933 | 1.518467 | 1.733417 | 1.664 | 1.63425 | 1.620417 | 1.535333 | 1.822767 | 1.718 |
| 3.5 | 1.64125 | 1.4744 | 1.61455 | 1.534817 | 1.501233 | 1.643383 | 1.6774 | 1.624317 | 1.680333 | 1.560083 | 1.655767 | 1.388717 | 1.6234 | 1.5368 | 1.500667 | 1.489883 | 1.405817 | 1.714533 | 1.599317 |
| 4 | 1.53805 | 1.362167 | 1.502517 | 1.416217 | 1.387333 | 1.539417 | 1.58145 | 1.525233 | 1.58155 | 1.45205 | 1.54865 | 1.27205 | 1.520367 | 1.420417 | 1.379933 | 1.37445 | 1.289833 | 1.614083 | 1.488367 |
| 4.5 | 1.441983 | 1.260433 | 1.400033 | 1.30975 | 1.28465 | 1.443367 | 1.491667 | 1.433933 | 1.489567 | 1.353617 | 1.448733 | 1.167367 | 1.423867 | 1.313783 | 1.271583 | 1.268833 | 1.186017 | 1.519417 | 1.383733 |
| 5 | 1.355 | 1.170867 | 1.30655 | 1.214967 | 1.19295 | 1.35385 | 1.408767 | 1.349633 | 1.403683 | 1.264167 | 1.356533 | 1.076533 | 1.3343 | 1.2179 | 1.176 | 1.176233 | 1.0953 | 1.431033 | 1.2895 |
| 5.5 | 1.275267 | 1.091733 | 1.22215 | 1.130967 | 1.11115 | 1.271967 | 1.331217 | 1.271667 | 1.323833 | 1.183283 | 1.2719 | 1.000633 | 1.251533 | 1.13265 | 1.092767 | 1.092883 | 1.018467 | 1.348867 | 1.204083 |
| 6 | 1.202133 | 1.02405 | 1.146367 | 1.05835 | 1.04005 | 1.196417 | 1.260117 | 1.200033 | 1.25055 | 1.111733 | 1.195633 | 0.938283 | 1.17685 | 1.059483 | 1.022517 | 1.023983 | 0.9541 | 1.272683 | 1.127767 |
| 6.5 | 1.136317 | 0.9665 | 1.079117 | 0.995783 | 0.9789 | 1.128767 | 1.19465 | 1.135183 | 1.183783 | 1.048517 | 1.127183 | 0.8877 | 1.109317 | 0.99635 | 0.963367 | 0.962417 | 0.90055 | 1.203183 | 1.06175 |
| 7 | 1.078083 | 0.9185 | 1.02015 | 0.942633 | 0.9273 | 1.06795 | 1.135417 | 1.077167 | 1.123467 | 0.994133 | 1.066433 | 0.84775 | 1.048983 | 0.943217 | 0.9147 | 0.913317 | 0.8575 | 1.139417 | 1.004717 |
| 7.5 | 1.02645 | 0.878883 | 0.969767 | 0.898933 | 0.884533 | 1.01515 | 1.082217 | 1.025917 | 1.069817 | 0.947633 | 1.0127 | 0.816183 | 0.996 | 0.898917 | 0.874667 | 0.870767 | 0.823783 | 1.0825 | 0.95545 |
| 8 | 0.9814 | 0.84675 | 0.92645 | 0.862783 | 0.849583 | 0.968983 | 1.034833 | 0.98155 | 1.0225 | 0.908317 | 0.966583 | 0.792367 | 0.950417 | 0.862917 | 0.842617 | 0.838483 | 0.797267 | 1.031483 | 0.914117 |
| 8.5 | 0.941183 | 0.820717 | 0.890117 | 0.833267 | 0.821 | 0.929433 | 0.9925 | 0.942383 | 0.9808 | 0.875217 | 0.926983 | 0.77385 | 0.910233 | 0.8334 | 0.816917 | 0.81015 | 0.77695 | 0.986333 | 0.879 |
| 9 | 0.907467 | 0.799817 | 0.859567 | 0.80915 | 0.798117 | 0.89505 | 0.955117 | 0.90895 | 0.94415 | 0.847583 | 0.893 | 0.7603 | 0.876433 | 0.80995 | 0.796733 | 0.789883 | 0.761283 | 0.945733 | 0.85 |
| 9.5 | 0.87765 | 0.783067 | 0.8341 | 0.789617 | 0.779617 | 0.865883 | 0.922117 | 0.8801 | 0.91245 | 0.82445 | 0.864733 | 0.749883 | 0.847 | 0.79125 | 0.78055 | 0.772 | 0.749683 | 0.91115 | 0.825783 |
| 10 | 0.8522 | 0.7699 | 0.813467 | 0.77385 | 0.765167 | 0.841167 | 0.8932 | 0.85535 | 0.884617 | 0.8055 | 0.84105 | 0.7422 | 0.822633 | 0.776483 | 0.7684 | 0.75965 | 0.74045 | 0.88035 | 0.8062 |
| 10.5 | 0.83055 | 0.759417 | 0.796383 | 0.7615 | 0.7536 | 0.82035 | 0.868083 | 0.833967 | 0.860983 | 0.790217 | 0.821083 | 0.736317 | 0.801617 | 0.764917 | 0.75895 | 0.748183 | 0.733883 | 0.854433 | 0.790083 |
| 11 | 0.813133 | 0.751233 | 0.7825 | 0.751317 | 0.744717 | 0.803017 | 0.846217 | 0.8164 | 0.84055 | 0.777667 | 0.804717 | 0.732167 | 0.784383 | 0.755983 | 0.751917 | 0.740817 | 0.728917 | 0.831667 | 0.77735 |
| 11.5 | 0.797283 | 0.744883 | 0.771267 | 0.7435 | 0.737817 | 0.788383 | 0.827433 | 0.80165 | 0.82325 | 0.7673 | 0.791483 | 0.728967 | 0.7698 | 0.749133 | 0.745833 | 0.733483 | 0.725183 | 0.812583 | 0.766767 |
| 12 | 0.785217 | 0.73985 | 0.762217 | 0.737483 | 0.732067 | 0.77625 | 0.811233 | 0.78895 | 0.8083 | 0.75915 | 0.78025 | 0.726667 | 0.758083 | 0.74365 | 0.74185 | 0.72955 | 0.7223 | 0.79635 | 0.75855 |
| 12.5 | 0.773667 | 0.73615 | 0.754983 | 0.733267 | 0.728167 | 0.766567 | 0.797617 | 0.7782 | 0.795883 | 0.752383 | 0.77145 | 0.724833 | 0.748483 | 0.739683 | 0.738867 | 0.725233 | 0.72045 | 0.78255 | 0.7515 |
| 13 | 0.765067 | 0.7331 | 0.74905 | 0.728733 | 0.724533 | 0.757967 | 0.785767 | 0.769733 | 0.785083 | 0.7472 | 0.764317 | 0.723867 | 0.740483 | 0.7366 | 0.736733 | 0.72295 | 0.71875 | 0.771017 | 0.7464 |
| 13.5 | 0.757867 | 0.730767 | 0.74445 | 0.72575 | 0.722033 | 0.751217 | 0.775717 | 0.76225 | 0.7763 | 0.742967 | 0.758483 | 0.723067 | 0.73415 | 0.734233 | 0.73465 | 0.720267 | 0.7178 | 0.761467 | 0.742033 |
| 14 | 0.751733 | 0.729133 | 0.740667 | 0.723183 | 0.71995 | 0.745433 | 0.767483 | 0.75625 | 0.7687 | 0.739567 | 0.753783 | 0.72255 | 0.728767 | 0.732517 | 0.7332 | 0.719333 | 0.717033 | 0.75335 | 0.73875 |
| 14.5 | 0.74625 | 0.72785 | 0.737667 | 0.721683 | 0.718467 | 0.740933 | 0.760367 | 0.75105 | 0.7624 | 0.73665 | 0.749867 | 0.721733 | 0.7244 | 0.731083 | 0.7325 | 0.717033 | 0.716517 | 0.74675 | 0.736083 |
| 15 | 0.74285 | 0.726817 | 0.73525 | 0.720017 | 0.717283 | 0.737017 | 0.754417 | 0.747283 | 0.757017 | 0.7345 | 0.746633 | 0.721817 | 0.721 | 0.72995 | 0.731783 | 0.716883 | 0.716333 | 0.741133 | 0.733983 |
| 15.5 | 0.738883 | 0.725817 | 0.7334 | 0.7196 | 0.7164 | 0.734183 | 0.7494 | 0.743717 | 0.7526 | 0.732683 | 0.7444 | 0.721217 | 0.718017 | 0.72915 | 0.7311 | 0.7155 | 0.715767 | 0.736567 | 0.732417 |
| 16 | 0.736317 | 0.7256 | 0.731983 | 0.718383 | 0.715667 | 0.731517 | 0.745317 | 0.74085 | 0.748683 | 0.7314 | 0.7423 | 0.721433 | 0.716017 | 0.728667 | 0.730817 | 0.715167 | 0.7154 | 0.732617 | 0.730967 |
| 16.5 | 0.733217 | 0.724717 | 0.730667 | 0.7181 | 0.715333 | 0.729383 | 0.741833 | 0.738467 | 0.745567 | 0.7302 | 0.740667 | 0.721167 | 0.713933 | 0.728017 | 0.73055 | 0.7145 | 0.7154 | 0.729667 | 0.73 |
| 17 | 0.7321 | 0.72505 | 0.72975 | 0.717367 | 0.714583 | 0.727767 | 0.738717 | 0.736733 | 0.743067 | 0.729267 | 0.739367 | 0.7214 | 0.712583 | 0.7278 | 0.7304 | 0.714967 | 0.715 | 0.727067 | 0.72915 |
| 17.5 | 0.73025 | 0.72425 | 0.72895 | 0.716867 | 0.7144 | 0.7263 | 0.73615 | 0.734667 | 0.740817 | 0.728467 | 0.73855 | 0.721017 | 0.711383 | 0.727467 | 0.73015 | 0.7139 | 0.71495 | 0.7251 | 0.72855 |
| 18 | 0.729067 | 0.72455 | 0.728333 | 0.716083 | 0.71395 | 0.725167 | 0.734033 | 0.733667 | 0.738883 | 0.728033 | 0.737633 | 0.721283 | 0.7104 | 0.7274 | 0.730017 | 0.713833 | 0.7146 | 0.723117 | 0.727917 |
| 18.5 | 0.7277 | 0.723933 | 0.727917 | 0.71655 | 0.71425 | 0.724383 | 0.7326 | 0.732933 | 0.7374 | 0.727417 | 0.736917 | 0.721217 | 0.70965 | 0.727233 | 0.7301 | 0.713967 | 0.7149 | 0.721617 | 0.727333 |
| 19 | 0.726917 | 0.723983 | 0.727483 | 0.7165 | 0.713817 | 0.723533 | 0.730833 | 0.7319 | 0.735967 | 0.7272 | 0.736567 | 0.721083 | 0.709083 | 0.7275 | 0.73005 | 0.714333 | 0.714967 | 0.720467 | 0.727183 |
| 19.5 | 0.7261 | 0.723733 | 0.727167 | 0.71655 | 0.713867 | 0.723033 | 0.729783 | 0.730883 | 0.734983 | 0.726667 | 0.736033 | 0.721583 | 0.708533 | 0.727233 | 0.730133 | 0.713767 | 0.714817 | 0.720167 | 0.726867 |
| 20 | 0.725317 | 0.723817 | 0.72705 | 0.716183 | 0.713733 | 0.722567 | 0.728633 | 0.730617 | 0.734067 | 0.726567 | 0.735867 | 0.721467 | 0.708367 | 0.727467 | 0.73 | 0.71405 | 0.714617 | 0.718683 | 0.726767 |

**Table 9.2 Plate reader data for LDH activity before freeze drying the screening investigation formulations detailed in Table 3.1**

| Run Number | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| 3.35745 | 3.34635 | 3.2583 | 3.279633 | 3.2632 | 3.01105 | 3.208667 | 3.101783 | 3.273967 | 3.032033 | 3.3038 | 3.3121 | 3.26035 | 3.30155 | 3.347917 | 3.232983 | 3.27205 | 3.143167 | 3.078 |
| 3.340167 | 3.319 | 3.1793 | 3.190717 | 3.181517 | 2.891783 | 3.13645 | 3.023967 | 3.2471 | 2.939467 | 3.210417 | 3.1945 | 3.171683 | 3.240567 | 3.317267 | 3.153733 | 3.15105 | 3.06095 | 2.944633 |
| 3.33255 | 3.304183 | 3.105267 | 3.1092 | 3.09405 | 2.790967 | 3.077667 | 2.95435 | 3.225033 | 2.850967 | 3.118233 | 3.068883 | 3.071583 | 3.18135 | 3.31055 | 3.088067 | 3.0431 | 2.9879 | 2.844833 |
| 3.320533 | 3.274517 | 3.020983 | 3.013017 | 3.00525 | 2.667567 | 3.006117 | 2.860133 | 3.198317 | 2.7581 | 3.007133 | 2.9299 | 2.965417 | 3.1096 | 3.277133 | 3.001133 | 2.9098 | 2.904 | 2.705917 |
| 3.310267 | 3.26085 | 2.940383 | 2.9283 | 2.91115 | 2.552033 | 2.946817 | 2.787417 | 3.180283 | 2.665883 | 2.8953 | 2.78155 | 2.851683 | 3.0338 | 3.253 | 2.912583 | 2.775633 | 2.812117 | 2.576417 |
| 3.293167 | 3.225883 | 2.85025 | 2.8272 | 2.813367 | 2.442033 | 2.86925 | 2.690717 | 3.153783 | 2.57015 | 2.7922 | 2.6478 | 2.747567 | 2.96405 | 3.229133 | 2.83715 | 2.654267 | 2.74105 | 2.46325 |
| 3.28185 | 3.207517 | 2.767233 | 2.73915 | 2.713617 | 2.342283 | 2.808933 | 2.618233 | 3.134083 | 2.479067 | 2.68955 | 2.513033 | 2.641283 | 2.89425 | 3.21555 | 2.760683 | 2.537667 | 2.663367 | 2.365967 |
| 3.263283 | 3.172017 | 2.672567 | 2.637933 | 2.6125 | 2.22335 | 2.73 | 2.520917 | 3.104083 | 2.382517 | 2.5759 | 2.376183 | 2.5318 | 2.813467 | 3.181017 | 2.666567 | 2.4065 | 2.577183 | 2.2307 |
| 3.2531 | 3.151583 | 2.5883 | 2.551067 | 2.512067 | 2.115867 | 2.66865 | 2.450333 | 3.085033 | 2.29305 | 2.465333 | 2.240917 | 2.4204 | 2.732467 | 3.15345 | 2.572433 | 2.2805 | 2.4825 | 2.111267 |
| 3.23135 | 3.113183 | 2.493933 | 2.4522 | 2.4116 | 2.01615 | 2.589033 | 2.353717 | 3.053133 | 2.198983 | 2.3678 | 2.128017 | 2.323217 | 2.6602 | 3.128283 | 2.496 | 2.175467 | 2.4135 | 2.015617 |
| 3.218283 | 3.091067 | 2.412183 | 2.372683 | 2.3141 | 1.9163 | 2.527567 | 2.286217 | 3.035433 | 2.113867 | 2.263617 | 2.0085 | 2.2186 | 2.578667 | 3.099017 | 2.401867 | 2.061917 | 2.320267 | 1.9027 |
| 3.194867 | 3.050283 | 2.319833 | 2.278367 | 2.21815 | 1.825017 | 2.44765 | 2.19325 | 3.000233 | 2.024117 | 2.173467 | 1.91465 | 2.129267 | 2.50715 | 3.072867 | 2.32775 | 1.9713 | 2.2542 | 1.814 |
| 3.179383 | 3.025783 | 2.241317 | 2.205733 | 2.125983 | 1.7355 | 2.387267 | 2.128967 | 2.978967 | 1.943417 | 2.078983 | 1.8153 | 2.034817 | 2.427433 | 3.042983 | 2.237433 | 1.87475 | 2.164683 | 1.715683 |
| 3.156633 | 2.9826 | 2.15545 | 2.121583 | 2.036617 | 1.654233 | 2.310317 | 2.042233 | 2.9467 | 1.86145 | 1.999533 | 1.740167 | 1.955667 | 2.35765 | 3.015167 | 2.1675 | 1.801417 | 2.101783 | 1.641883 |
| 3.13915 | 2.956467 | 2.08315 | 2.0552 | 1.95545 | 1.57555 | 2.250533 | 1.983067 | 2.9226 | 1.786967 | 1.915733 | 1.661033 | 1.8711 | 2.281217 | 2.985567 | 2.0829 | 1.720433 | 2.016333 | 1.555283 |
| 3.114517 | 2.913733 | 2.003733 | 1.97735 | 1.876533 | 1.50535 | 2.175183 | 1.901667 | 2.889433 | 1.709917 | 1.848517 | 1.605883 | 1.8033 | 2.21505 | 2.956683 | 2.017317 | 1.662583 | 1.959 | 1.49475 |
| 3.0983 | 2.88665 | 1.938917 | 1.919633 | 1.80505 | 1.451333 | 2.11995 | 1.849817 | 2.868133 | 1.64405 | 1.788017 | 1.554817 | 1.739233 | 2.152 | 2.940833 | 1.956883 | 1.61575 | 1.897267 | 1.453433 |
| 3.072517 | 2.840983 | 1.868333 | 1.852683 | 1.737483 | 1.378317 | 2.049217 | 1.7745 | 2.833883 | 1.577483 | 1.72085 | 1.5079 | 1.674067 | 2.0809 | 2.896483 | 1.879817 | 1.5561 | 1.827483 | 1.3767 |
| 3.055033 | 2.81345 | 1.812183 | 1.802783 | 1.677083 | 1.322117 | 1.998367 | 1.72785 | 2.808667 | 1.517833 | 1.66145 | 1.462667 | 1.613317 | 2.012967 | 2.864083 | 1.807417 | 1.50535 | 1.753967 | 1.315333 |
| 3.027517 | 2.76615 | 1.749283 | 1.742733 | 1.6202 | 1.274183 | 1.930383 | 1.6595 | 2.7742 | 1.458033 | 1.616117 | 1.4391 | 1.56935 | 1.957383 | 2.8348 | 1.756417 | 1.476483 | 1.708517 | 1.28095 |
| 3.008833 | 2.7376 | 1.701617 | 1.700383 | 1.570533 | 1.228667 | 1.883967 | 1.61985 | 2.7497 | 1.40785 | 1.567683 | 1.406533 | 1.520083 | 1.893483 | 2.80315 | 1.691767 | 1.438133 | 1.640617 | 1.232083 |
| 2.981867 | 2.691717 | 1.64735 | 1.6474 | 1.525133 | 1.19215 | 1.821567 | 1.55755 | 2.716933 | 1.357367 | 1.533917 | 1.393433 | 1.48715 | 1.84475 | 2.772567 | 1.6474 | 1.419767 | 1.601317 | 1.208367 |
| 2.963067 | 2.663683 | 1.607617 | 1.6136 | 1.486183 | 1.156183 | 1.77845 | 1.5236 | 2.692617 | 1.314833 | 1.4954 | 1.370133 | 1.447817 | 1.788767 | 2.740217 | 1.5914 | 1.391317 | 1.54035 | 1.170417 |
| 2.933917 | 2.615517 | 1.5617 | 1.567 | 1.4502 | 1.128333 | 1.719683 | 1.46795 | 2.656833 | 1.271633 | 1.47015 | 1.36495 | 1.4245 | 1.744217 | 2.7095 | 1.554133 | 1.3813 | 1.508967 | 1.154983 |
| 2.9148 | 2.588217 | 1.5294 | 1.539583 | 1.4204 | 1.100917 | 1.684583 | 1.44105 | 2.63535 | 1.23665 | 1.440433 | 1.348783 | 1.393733 | 1.695117 | 2.677033 | 1.504517 | 1.35895 | 1.45375 | 1.1233 |
| 2.885633 | 2.541767 | 1.490967 | 1.501533 | 1.394017 | 1.080817 | 1.63095 | 1.391567 | 2.6007 | 1.200983 | 1.4233 | 1.347383 | 1.3773 | 1.65745 | 2.646867 | 1.475683 | 1.356167 | 1.428067 | 1.118 |
| 2.866133 | 2.513717 | 1.465483 | 1.478483 | 1.372083 | 1.0598 | 1.59805 | 1.370517 | 2.577467 | 1.1739 | 1.40045 | 1.334033 | 1.353167 | 1.61205 | 2.613783 | 1.43265 | 1.338983 | 1.3798 | 1.091667 |
| 2.8373 | 2.467167 | 1.433533 | 1.445117 | 1.352383 | 1.045833 | 1.54795 | 1.32635 | 2.54435 | 1.14565 | 1.389283 | 1.337033 | 1.34395 | 1.582483 | 2.584633 | 1.410567 | 1.338633 | 1.360533 | 1.092333 |
| 2.817383 | 2.439617 | 1.413933 | 1.429683 | 1.336067 | 1.029767 | 1.523317 | 1.3112 | 2.522433 | 1.124517 | 1.371833 | 1.326233 | 1.325567 | 1.545117 | 2.551133 | 1.373117 | 1.3256 | 1.3175 | 1.069733 |
| 2.787967 | 2.394483 | 1.388167 | 1.402667 | 1.322983 | 1.02125 | 1.480067 | 1.272567 | 2.4886 | 1.101417 | 1.36465 | 1.330833 | 1.320667 | 1.5194 | 2.522767 | 1.35625 | 1.327267 | 1.303817 | 1.072283 |
| 2.767933 | 2.367267 | 1.375267 | 1.392233 | 1.3117 | 1.009617 | 1.4554 | 1.262683 | 2.466117 | 1.08605 | 1.351567 | 1.321817 | 1.306267 | 1.4872 | 2.490333 | 1.325417 | 1.316933 | 1.266483 | 1.056217 |
| 2.738467 | 2.321633 | 1.353467 | 1.3686 | 1.3013 | 1.003933 | 1.416055 | 1.227733 | 2.431117 | 1.067133 | 1.348067 | 1.328017 | 1.304483 | 1.467017 | 2.46125 | 1.31355 | 1.321467 | 1.257467 | 1.0614 |
| 2.7185 | 2.296433 | 1.345483 | 1.362 | 1.294667 | 0.996183 | 1.4003 | 1.22205 | 2.409533 | 1.05685 | 1.337517 | 1.3199 | 1.293983 | 1.4388 | 2.4294 | 1.287583 | 1.311733 | 1.225267 | 1.047817 |
| 2.6898 | 2.2522 | 1.327783 | 1.34055 | 1.287433 | 0.992717 | 1.3661 | 1.1916 | 2.377567 | 1.04335 | 1.337133 | 1.32635 | 1.29435 | 1.42345 | 2.401317 | 1.2807 | 1.318767 | 1.221283 | 1.055783 |
| 2.667967 | 2.227567 | 1.3226 | 1.338433 | 1.283467 | 0.986633 | 1.3515 | 1.189667 | 2.356067 | 1.036117 | 1.328167 | 1.3198 | 1.285333 | 1.398917 | 2.3703 | 1.258017 | 1.310483 | 1.1922 | 1.04285 |
| 2.638783 | 2.185367 | 1.308833 | 1.322433 | 1.2786 | 0.9849 | 1.3228 | 1.163167 | 2.3254 | 1.025483 | 1.329533 | 1.325883 | 1.287933 | 1.388133 | 2.341617 | 1.254433 | 1.316733 | 1.192083 | 1.04925 |
| 2.6198 | 2.161667 | 1.30645 | 1.321367 | 1.2765 | 0.98105 | 1.312283 | 1.165183 | 2.303683 | 1.021617 | 1.3222 | 1.319167 | 1.2797 | 1.369283 | 2.311633 | 1.234567 | 1.3076 | 1.166517 | 1.039483 |
| 2.59045 | 2.120267 | 1.294633 | 1.307667 | 1.27315 | 0.9812 | 1.2842 | 1.141717 | 2.275017 | 1.0126 | 1.324717 | 1.3258 | 1.283467 | 1.360217 | 2.283783 | 1.23485 | 1.315033 | 1.169283 | 1.0477 |
| 2.570567 | 2.098817 | 1.294667 | 1.308733 | 1.271917 | 0.991833 | 1.27805 | 1.146367 | 2.254517 | 1.0102 | 1.32905 | 1.32955 | 1.283883 | 1.353983 | 2.26945 | 1.236367 | 1.32455 | 1.166133 | 1.068567 |
| 2.541833 | 2.058033 | 1.28475 | 1.297133 | 1.270117 | 0.97785 | 1.256917 | 1.125083 | 2.2243 | 1.003267 | 1.321983 | 1.326383 | 1.28075 | 1.3382 | 2.2272 | 1.219883 | 1.314133 | 1.152217 | 1.047733 |
| 2.522133 | 2.037317 | 1.286917 | 1.299333 | 1.269967 | 0.9898 | 1.250967 | 1.13155 | 2.2045 | 1.002683 | 1.327017 | 1.330417 | 1.281433 | 1.33455 | 2.214 | 1.222833 | 1.323817 | 1.1513 | 1.067417 |

**Table 9.3 Plate reader data for LDH activity after freeze drying the screening investigation formulations detailed in Table 3.1**

| Time (mins) | Run Number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 0 | 2.463217 | 2.4896 | 2.4808 | 2.451033 | 2.301275 | 2.406217 | 2.411567 | 2.384483 | 2.500383 | 2.4737 | 2.632833 |
| 0.5 | 2.340033 | 2.341117 | 2.349367 | 2.33085 | 2.165925 | 2.286167 | 2.29285 | 2.265033 | 2.41685 | 2.377267 | 2.553767 |
| 1 | 2.212383 | 2.219383 | 2.218417 | 2.2097 | 2.029125 | 2.159 | 2.166067 | 2.13645 | 2.326883 | 2.296017 | 2.4801 |
| 1.5 | 2.090717 | 2.096967 | 2.092767 | 2.090933 | 1.89305 | 2.033683 | 2.043517 | 2.010283 | 2.238383 | 2.204933 | 2.409933 |
| 2 | 1.971917 | 1.974817 | 1.97455 | 1.973533 | 1.7616 | 1.9124 | 1.924333 | 1.8895 | 2.149617 | 2.11165 | 2.34155 |
| 2.5 | 1.85465 | 1.857717 | 1.860833 | 1.859783 | 1.63525 | 1.794817 | 1.8095 | 1.7722 | 2.063717 | 2.020617 | 2.275233 |
| 3 | 1.742083 | 1.74785 | 1.748583 | 1.750983 | 1.5147 | 1.680617 | 1.6982 | 1.659433 | 1.97805 | 1.932133 | 2.21025 |
| 3.5 | 1.633983 | 1.6409 | 1.6415 | 1.644467 | 1.402025 | 1.5699 | 1.5903 | 1.551067 | 1.893217 | 1.846917 | 2.145767 |
| 4 | 1.52895 | 1.53765 | 1.539417 | 1.541283 | 1.29475 | 1.46685 | 1.487733 | 1.448667 | 1.811483 | 1.764867 | 2.080167 |
| 4.5 | 1.429933 | 1.438917 | 1.441217 | 1.4435 | 1.1936 | 1.369083 | 1.390233 | 1.352167 | 1.732283 | 1.684967 | 2.0156 |
| 5 | 1.33655 | 1.34615 | 1.347817 | 1.351633 | 1.100825 | 1.27715 | 1.298583 | 1.261517 | 1.65675 | 1.60755 | 1.952083 |
| 5.5 | 1.248933 | 1.259033 | 1.26115 | 1.265833 | 1.015725 | 1.192333 | 1.212283 | 1.17735 | 1.583067 | 1.532983 | 1.888817 |
| 6 | 1.16755 | 1.177833 | 1.1802 | 1.18385 | 0.937325 | 1.11405 | 1.13285 | 1.100267 | 1.512233 | 1.461217 | 1.825667 |
| 6.5 | 1.093217 | 1.102817 | 1.1045 | 1.109133 | 0.867475 | 1.042583 | 1.06015 | 1.0295 | 1.44445 | 1.393567 | 1.763733 |
| 7 | 1.024883 | 1.0345 | 1.035267 | 1.0409 | 0.806525 | 0.977417 | 0.99335 | 0.966 | 1.378533 | 1.32935 | 1.7044 |
| 7.5 | 0.963233 | 0.9731 | 0.973733 | 0.978517 | 0.753375 | 0.920333 | 0.934 | 0.9096 | 1.317367 | 1.269217 | 1.645567 |
| 8 | 0.908383 | 0.9176 | 0.9192 | 0.921967 | 0.70725 | 0.869567 | 0.881083 | 0.8602 | 1.261483 | 1.2133 | 1.5881 |
| 8.5 | 0.859467 | 0.868483 | 0.870433 | 0.871867 | 0.6678 | 0.825617 | 0.83445 | 0.81645 | 1.210533 | 1.1608 | 1.532483 |
| 9 | 0.816383 | 0.82515 | 0.827567 | 0.828417 | 0.63545 | 0.787133 | 0.79435 | 0.779133 | 1.161683 | 1.1116 | 1.47895 |
| 9.5 | 0.778983 | 0.788067 | 0.790167 | 0.790017 | 0.6088 | 0.75445 | 0.7588 | 0.746667 | 1.114933 | 1.065167 | 1.42705 |
| 10 | 0.746383 | 0.756033 | 0.75725 | 0.755167 | 0.58665 | 0.726817 | 0.728017 | 0.719883 | 1.07035 | 1.021517 | 1.376967 |
| 10.5 | 0.718233 | 0.728617 | 0.72865 | 0.726417 | 0.56875 | 0.70335 | 0.702867 | 0.697233 | 1.027917 | 0.981333 | 1.3293 |
| 11 | 0.694417 | 0.70515 | 0.704183 | 0.701467 | 0.55435 | 0.683683 | 0.6811 | 0.677817 | 0.989233 | 0.944367 | 1.283367 |
| 11.5 | 0.674167 | 0.68545 | 0.684167 | 0.680383 | 0.5429 | 0.667233 | 0.66305 | 0.662167 | 0.952767 | 0.911333 | 1.239183 |
| 12 | 0.656933 | 0.668483 | 0.666933 | 0.662467 | 0.533075 | 0.654183 | 0.6478 | 0.649533 | 0.919967 | 0.8796 | 1.19735 |
| 12.5 | 0.64225 | 0.654283 | 0.652183 | 0.647483 | 0.525125 | 0.6429 | 0.6354 | 0.6392 | 0.888983 | 0.849817 | 1.157733 |
| 13 | 0.630167 | 0.642517 | 0.640183 | 0.635783 | 0.51915 | 0.633767 | 0.62525 | 0.630217 | 0.860117 | 0.822417 | 1.1203 |
| 13.5 | 0.619867 | 0.63285 | 0.630717 | 0.625283 | 0.51445 | 0.62645 | 0.6166 | 0.62365 | 0.8338 | 0.7974 | 1.08425 |
| 14 | 0.61175 | 0.624533 | 0.62225 | 0.616567 | 0.509825 | 0.6203 | 0.609733 | 0.617883 | 0.811883 | 0.775883 | 1.0489 |
| 14.5 | 0.60485 | 0.617683 | 0.61535 | 0.6097 | 0.506825 | 0.615183 | 0.604167 | 0.613467 | 0.793317 | 0.756567 | 1.015033 |
| 15 | 0.599033 | 0.6123 | 0.609867 | 0.604267 | 0.50465 | 0.610767 | 0.599767 | 0.609517 | 0.775717 | 0.739617 | 0.98325 |
| 15.5 | 0.594417 | 0.608083 | 0.605683 | 0.5994 | 0.5027 | 0.607433 | 0.5957 | 0.606383 | 0.758983 | 0.724617 | 0.9536 |
| 16 | 0.59075 | 0.604333 | 0.6019 | 0.595617 | 0.500475 | 0.605067 | 0.592667 | 0.603917 | 0.743733 | 0.70975 | 0.927183 |
| 16.5 | 0.587683 | 0.60135 | 0.598533 | 0.592517 | 0.498975 | 0.602733 | 0.590267 | 0.60215 | 0.729717 | 0.696933 | 0.903517 |
| 17 | 0.585167 | 0.5991 | 0.59605 | 0.590267 | 0.498475 | 0.600983 | 0.5884 | 0.600083 | 0.718167 | 0.686067 | 0.88215 |
| 17.5 | 0.583067 | 0.597267 | 0.5937 | 0.587983 | 0.49765 | 0.599117 | 0.586533 | 0.599033 | 0.706367 | 0.676283 | 0.862267 |
| 18 | 0.581283 | 0.595683 | 0.592183 | 0.586283 | 0.496825 | 0.5981 | 0.585483 | 0.598067 | 0.69695 | 0.668033 | 0.844017 |
| 18.5 | 0.579717 | 0.594217 | 0.590467 | 0.585267 | 0.49605 | 0.596817 | 0.5845 | 0.596967 | 0.6873 | 0.660467 | 0.827283 |
| 19 | 0.578667 | 0.59335 | 0.589 | 0.584167 | 0.4959 | 0.595883 | 0.583767 | 0.596417 | 0.680033 | 0.653817 | 0.8107 |
| 19.5 | 0.577567 | 0.5923 | 0.588133 | 0.583 | 0.495475 | 0.595083 | 0.5829 | 0.595933 | 0.672383 | 0.648 | 0.793067 |
| 20 | 0.57665 | 0.591583 | 0.58735 | 0.582217 | 0.49495 | 0.5944 | 0.58215 | 0.595083 | 0.665417 | 0.643033 | 0.776017 |

**Table 9.4 Plate reader data for LDH activity before freeze drying the optimization investigation formulations detailed in Table 3.2**

| Time (mins) | Run Number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 0 | 3.152567 | 3.120267 | 3.136217 | 3.110567 | 3.074417 | 3.371117 | 3.024267 | 3.28585 | 3.207767 | 3.283783 | 0.292283 |
| 0.5 | 3.0801 | 3.028183 | 3.034583 | 3.023583 | 2.987517 | 3.342517 | 2.917983 | 3.24455 | 3.200667 | 3.292633 | 0.2928 |
| 1 | 2.986317 | 2.92425 | 2.930283 | 2.928517 | 2.893367 | 3.309333 | 2.80775 | 3.193783 | 3.1893 | 3.2869 | 0.308683 |
| 1.5 | 2.898683 | 2.8265 | 2.8183 | 2.8354 | 2.791533 | 3.277983 | 2.6965 | 3.147217 | 3.177833 | 3.296467 | 0.301883 |
| 2 | 2.800317 | 2.7162 | 2.71515 | 2.745167 | 2.69205 | 3.23225 | 2.5948 | 3.0968 | 3.178117 | 3.289617 | 0.319133 |
| 2.5 | 2.707283 | 2.612583 | 2.608583 | 2.657217 | 2.592267 | 3.197533 | 2.492117 | 3.046 | 3.158333 | 3.29835 | 0.316767 |
| 3 | 2.601617 | 2.51485 | 2.498717 | 2.5516 | 2.48435 | 3.1534 | 2.379567 | 2.9876 | 3.146633 | 3.285817 | 0.325617 |
| 3.5 | 2.488433 | 2.389067 | 2.378867 | 2.4219 | 2.366667 | 3.115617 | 2.257117 | 2.925617 | 3.128867 | 3.282283 | 0.32925 |
| 4 | 2.359333 | 2.26025 | 2.2408 | 2.297333 | 2.240683 | 3.0696 | 2.12925 | 2.8655 | 3.123683 | 3.2752 | 0.328733 |
| 4.5 | 2.25115 | 2.153383 | 2.13155 | 2.2003 | 2.134017 | 3.0291 | 2.035 | 2.809183 | 3.109383 | 3.275033 | 0.327117 |
| 5 | 2.150467 | 2.066017 | 2.036833 | 2.111383 | 2.040333 | 2.982633 | 1.9451 | 2.75135 | 3.099367 | 3.28305 | 0.353217 |
| 5.5 | 2.07295 | 1.982183 | 1.953417 | 2.043783 | 1.96485 | 2.938967 | 1.880567 | 2.694583 | 3.090717 | 3.28835 | 0.254717 |
| 6 | 1.99565 | 1.904467 | 1.879217 | 1.977633 | 1.8908 | 2.888433 | 1.814633 | 2.637667 | 3.073617 | 3.269083 | 0.292733 |
| 6.5 | 1.89925 | 1.8197 | 1.794117 | 1.87755 | 1.8 | 2.831933 | 1.724967 | 2.573983 | 3.050433 | 3.263617 | 0.263367 |
| 7 | 1.806683 | 1.7326 | 1.6964 | 1.776167 | 1.7055 | 2.777417 | 1.633617 | 2.5116 | 3.037067 | 3.255 | 0.285067 |
| 7.5 | 1.725867 | 1.6515 | 1.621483 | 1.70125 | 1.631333 | 2.724733 | 1.566017 | 2.451283 | 3.020517 | 3.257367 | 0.267033 |
| 8 | 1.665717 | 1.600083 | 1.5616 | 1.647317 | 1.570717 | 2.671517 | 1.51465 | 2.3981 | 3.00815 | 3.2529 | 0.299 |
| 8.5 | 1.60845 | 1.555933 | 1.515933 | 1.597317 | 1.528383 | 2.622933 | 1.4732 | 2.351517 | 2.998617 | 3.2525 | 0.272883 |
| 9 | 1.571933 | 1.5269 | 1.482467 | 1.572733 | 1.5002 | 2.575967 | 1.45235 | 2.3023 | 2.993667 | 3.2539 | 0.30545 |
| 9.5 | 1.541083 | 1.4947 | 1.464683 | 1.545983 | 1.475033 | 2.52955 | 1.428733 | 2.258883 | 2.974983 | 3.261717 | 0.287033 |
| 10 | 1.5093 | 1.461217 | 1.434917 | 1.5031 | 1.445283 | 2.474483 | 1.399483 | 2.205017 | 2.9536 | 3.236717 | 0.289733 |
| 10.5 | 1.464833 | 1.422833 | 1.386483 | 1.44195 | 1.396583 | 2.415533 | 1.336733 | 2.147983 | 2.93555 | 3.225433 | 0.286317 |
| 11 | 1.41525 | 1.38505 | 1.3422 | 1.393233 | 1.354017 | 2.36395 | 1.310217 | 2.0993 | 2.918817 | 3.232683 | 0.29835 |
| 11.5 | 1.390267 | 1.355933 | 1.32945 | 1.365233 | 1.3346 | 2.314967 | 1.28145 | 2.05765 | 2.89875 | 3.228117 | 0.284817 |
| 12 | 1.37045 | 1.3545 | 1.306967 | 1.357433 | 1.318383 | 2.2715 | 1.269333 | 2.018617 | 2.886783 | 3.209267 | 0.298283 |
| 12.5 | 1.358417 | 1.349517 | 1.3005 | 1.34635 | 1.311517 | 2.229883 | 1.259267 | 1.984483 | 2.873867 | 3.221283 | 0.283417 |
| 13 | 1.355267 | 1.351433 | 1.303 | 1.354167 | 1.313683 | 2.1918 | 1.261667 | 1.957933 | 2.866983 | 3.2226 | 0.261167 |
| 13.5 | 1.365617 | 1.349333 | 1.313133 | 1.359833 | 1.325017 | 2.1564 | 1.274733 | 1.929683 | 2.85965 | 3.214933 | 0.2658 |
| 14 | 1.363933 | 1.358867 | 1.318233 | 1.3661 | 1.329417 | 2.119217 | 1.2794 | 1.898667 | 2.84205 | 3.2149 | 0.283883 |
| 14.5 | 1.359367 | 1.342067 | 1.310367 | 1.3369 | 1.323567 | 2.07245 | 1.256783 | 1.861133 | 2.816167 | 3.209517 | 0.290267 |
| 15 | 1.345 | 1.321833 | 1.2886 | 1.3121 | 1.295483 | 2.0309 | 1.233 | 1.828983 | 2.794333 | 3.190083 | 0.254933 |
| 15.5 | 1.323317 | 1.309333 | 1.275283 | 1.299283 | 1.28415 | 1.9927 | 1.218533 | 1.79955 | 2.773117 | 3.179917 | 0.27625 |
| 16 | 1.319383 | 1.3071 | 1.272633 | 1.288167 | 1.281033 | 1.9581 | 1.213617 | 1.7779 | 2.761483 | 3.194367 | 0.2919 |
| 16.5 | 1.312283 | 1.302917 | 1.2679 | 1.28935 | 1.277567 | 1.927583 | 1.21635 | 1.757717 | 2.747433 | 3.19115 | 0.26345 |
| 17 | 1.320983 | 1.308417 | 1.272883 | 1.300117 | 1.279117 | 1.89905 | 1.2135 | 1.74035 | 2.738367 | 3.18905 | 0.283067 |
| 17.5 | 1.32045 | 1.318633 | 1.275717 | 1.306467 | 1.29035 | 1.873967 | 1.2226 | 1.727383 | 2.726583 | 3.16915 | 0.26455 |
| 18 | 1.3292 | 1.331433 | 1.288717 | 1.324783 | 1.303267 | 1.8524 | 1.239417 | 1.7175 | 2.7156 | 3.1863 | 0.265717 |
| 18.5 | 1.34425 | 1.337733 | 1.301583 | 1.34325 | 1.32115 | 1.831317 | 1.255383 | 1.707167 | 2.7133 | 3.170333 | 0.276 |
| 19 | 1.350417 | 1.349783 | 1.310333 | 1.35535 | 1.3319 | 1.810967 | 1.274883 | 1.691617 | 2.6914 | 3.169883 | 0.290267 |
| 19.5 | 1.349067 | 1.3423 | 1.308833 | 1.3304 | 1.325967 | 1.78015 | 1.261283 | 1.6709 | 2.671433 | 3.157033 | 0.266767 |
| 20 | 1.335283 | 1.321233 | 1.293683 | 1.307467 | 1.303383 | 1.750117 | 1.22885 | 1.653467 | 2.65235 | 3.15715 | 0.255717 |

**Table 9.5 Plate reader data for LDH activity after freeze drying the optimization investigation formulations detailed in Table 3.2**

| PG/ML | Control | 15 | 16 | 14 | 11 | 1 |
|---|---|---|---|---|---|---|
| 500 | 14208 | 22739 | 10006 | 6610 | 7556 | 26774 |
| 250.0 | 10351 | 11746 | 5550 | 3906 | 3006 | 14824 |
| 125.0 | 6457 | 5899 | 3062 | 2197 | 1285 | 7940 |
| 62.5 | 3223 | 2746 | 1627 | 1126 | 624 | 3620 |
| 31.3 | 1630 | 1385 | 967 | 631 | 314 | 1646 |
| 15.6 | 778 | 606 | 464 | 304 | 201 | 760 |
| 7.8 | 425 | 336 | 286 | 203 | 164 | 437 |
| 3.9 | 222 | 194 | 175 | 138 | 118 | 283 |
| | | | | | | |
| PG/ML | 3 | Control | 2 | 10 | 18 | 9 |
| 500 | 27925 | 21189 | 442 | 24545 | 30163 | 13949 |
| 250.0 | 14954 | 15472 | 275 | 14371 | 16503 | 9379 |
| 125.0 | 7185 | 9750 | 249 | 8031 | 7451 | 4969 |
| 62.5 | 3215 | 5417 | 209 | 3896 | 3160 | 2390 |
| 31.3 | 1446 | 2852 | 169 | 2071 | 1482 | 1055 |
| 15.6 | 662 | 1434 | 161 | 964 | 738 | 620 |
| 7.8 | 341 | 761 | 133 | 472 | 387 | 326 |
| 3.9 | 202 | 358 | 123 | 253 | 251 | 204 |
| | | | | | | |
| PG/ML | 6 | 12 | Control | 17 | 13 | 4 |
| 500 | 223 | 589 | 22703 | 780 | 33561 | 37766 |
| 250.0 | 153 | 362 | 17719 | 451 | 21542 | 21648 |
| 125.0 | 118 | 210 | 10181 | 295 | 10355 | 9799 |
| 62.5 | 106 | 168 | 6056 | 223 | 4727 | 4444 |
| 31.3 | 97 | 177 | 3680 | 208 | 2184 | 1825 |
| 15.6 | 102 | 142 | 1794 | 171 | 1016 | 968 |
| 7.8 | 79 | 127 | 883 | 147 | 503 | 453 |
| 3.9 | 90 | 110 | 452 | 121 | 302 | 222 |
| | | | | | | |
| PG/ML | 5 | 8 | 19 | Control | 7 | 20 |
| 500 | 28743 | 30417 | 10253 | 30214 | 2727 | 29534 |
| 250.0 | 15383 | 17518 | 5215 | 19629 | 1062 | 16682 |
| 125.0 | 6840 | 8672 | 2406 | 12877 | 509 | 8278 |
| 62.5 | 3002 | 3685 | 1224 | 6271 | 293 | 4146 |
| 31.3 | 1351 | 1852 | 701 | 3227 | 239 | 1817 |
| 15.6 | 616 | 795 | 388 | 1722 | 157 | 727 |
| 7.8 | 344 | 412 | 256 | 765 | 152 | 376 |
| 3.9 | 206 | 249 | 164 | 338 | 107 | 211 |

**Table 9.6 Example of cell count data obtained from G-csf assay. This table shows the mean cell counts of one assay conducted on the initial formulation screen the recipes for which can be found in Table 4.2. The control was an unformulated sample that was not freeze dried**

| Run | 1 | | | | Run | 2 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.712 | 10.7536 | 0.0419 | | 1 | 10.5471 | 10.5783 | 0.0312 |
| 2 | 10.73 | 10.7708 | 0.041 | | 2 | 10.6697 | 10.7005 | 0.0308 |
| 3 | 10.767 | 10.8082 | 0.0416 | | 3 | 10.6653 | 10.6963 | 0.031 |
| 4 | 10.667 | 10.7082 | 0.0415 | | 4 | 10.7137 | 10.7446 | 0.0309 |
| 5 | 10.528 | 10.5692 | 0.0413 | | 5 | 10.5451 | 10.576 | 0.0309 |
| 6 | 10.707 | 10.7488 | 0.0415 | | 6 | 10.5924 | 10.6235 | 0.0311 |
| 7 | 10.817 | 10.8578 | 0.0411 | | 7 | 10.7093 | 10.7403 | 0.031 |
| 8 | 10.646 | 10.6881 | 0.0419 | | 8 | 10.5213 | 10.5523 | 0.031 |
| 9 | 10.672 | 10.7139 | 0.0417 | | 9 | 10.6958 | 10.7268 | 0.031 |
| 10 | 10.624 | 10.6537 | 0.0302 | | 10 | 10.5691 | 10.5998 | 0.0307 |
| | | | | | | | | |
| Run | 3 | | | | Run | 4 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.683 | 10.714 | 0.0309 | | 1 | 10.794 | 10.8194 | 0.0254 |
| 2 | 10.715 | 10.7459 | 0.0311 | | 2 | 10.558 | 10.5743 | 0.0163 |
| 3 | 10.627 | 10.6584 | 0.0318 | | 3 | 10.6868 | 10.7133 | 0.0265 |
| 4 | 10.746 | 10.7773 | 0.0309 | | 4 | 10.5954 | 10.6208 | 0.0254 |
| 5 | 10.715 | 10.746 | 0.031 | | 5 | 10.4827 | 10.6069 | 0.1242 |
| 6 | 10.622 | 10.6524 | 0.0302 | | 6 | 10.5545 | 10.5806 | 0.0261 |
| 7 | 10.648 | 10.6755 | 0.0275 | | 7 | 10.5249 | 10.5503 | 0.0254 |
| 8 | 10.767 | 10.7985 | 0.0313 | | 8 | 10.6973 | 10.7233 | 0.026 |
| 9 | 10.585 | 10.6152 | 0.0299 | | 9 | 10.537 | 10.5619 | 0.0249 |
| 10 | 10.566 | 10.5982 | 0.0325 | | 10 | 10.5483 | 10.5683 | 0.02 |
| | | | | | | | | |
| Run | 5 | | | | Run | 6 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.626 | 10.6606 | 0.0348 | | 1 | 10.6589 | 10.6842 | 0.0253 |
| 2 | 10.653 | | -10.653 | | 2 | 10.6022 | 10.6274 | 0.0252 |
| 3 | 10.746 | 10.7808 | 0.0352 | | 3 | 10.7105 | 10.7356 | 0.0251 |
| 4 | 10.694 | | -10.694 | | 4 | 10.6296 | 10.6549 | 0.0253 |
| 5 | 10.72 | | -10.72 | | 5 | 10.732 | 10.7574 | 0.0254 |
| 6 | 10.56 | | -10.56 | | 6 | 10.5114 | 10.5365 | 0.0251 |
| 7 | 10.581 | | -10.581 | | 7 | 10.6702 | 10.6955 | 0.0253 |
| 8 | 10.657 | 10.6914 | 0.0348 | | 8 | 10.5153 | 10.5409 | 0.0256 |
| 9 | 10.569 | | -10.569 | | 9 | 10.5403 | 10.5662 | 0.0259 |
| 10 | 10.679 | | -10.679 | | 10 | 10.7432 | 10.7682 | 0.025 |

**Table 9.7 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [1/10]**

| Run | 7 | | | | Run | 8 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.693 | 10.7205 | 0.0274 | | 1 | 10.6835 | 10.7164 | 0.0329 |
| 2 | 10.635 | 10.6628 | 0.0274 | | 2 | 10.7531 | 10.7856 | 0.0325 |
| 3 | 10.747 | 10.772 | 0.0254 | | 3 | 10.6589 | 10.6918 | 0.0329 |
| 4 | 10.672 | 10.6991 | 0.0273 | | 4 | 10.6353 | 10.6678 | 0.0325 |
| 5 | 10.763 | 10.7898 | 0.0273 | | 5 | 10.5257 | 10.5558 | 0.0301 |
| 6 | 10.676 | 10.7049 | 0.0286 | | 6 | 10.6286 | 10.6606 | 0.032 |
| 7 | 10.69 | 10.7171 | 0.027 | | 7 | 10.7067 | 10.7384 | 0.0317 |
| 8 | 10.639 | 10.6655 | 0.027 | | 8 | 10.7293 | 10.7603 | 0.031 |
| 9 | 10.669 | 10.6958 | 0.0272 | | 9 | 10.5408 | 10.5711 | 0.0303 |
| 10 | 10.677 | 10.6983 | 0.0215 | | 10 | 10.5901 | 10.617 | 0.0269 |
| | | | | | | | | |
| Run | 9 | | | | Run | 10 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.692 | 10.718 | 0.0265 | | 1 | 10.7318 | 10.7625 | 0.0307 |
| 2 | 10.671 | 10.6975 | 0.0262 | | 2 | 10.7181 | 10.7489 | 0.0308 |
| 3 | 10.707 | 10.7326 | 0.0261 | | 3 | 10.7203 | 10.7508 | 0.0305 |
| 4 | 10.751 | 10.777 | 0.0264 | | 4 | 10.7157 | 10.7459 | 0.0302 |
| 5 | 10.721 | 10.7464 | 0.0258 | | 5 | 10.6801 | 10.7107 | 0.0306 |
| 6 | 10.65 | 10.6764 | 0.0263 | | 6 | 10.7333 | 10.7631 | 0.0298 |
| 7 | 10.713 | 10.7388 | 0.0257 | | 7 | 10.6007 | 10.6909 | 0.0902 |
| 8 | 10.765 | 10.7907 | 0.0262 | | 8 | 10.6531 | 10.6831 | 0.03 |
| 9 | 10.691 | 10.7168 | 0.0259 | | 9 | 10.5897 | 10.62 | 0.0303 |
| 10 | 10.638 | 10.6677 | 0.0298 | | 10 | 10.7018 | 10.7351 | 0.0333 |
| | | | | | | | | |
| Run | 11 | | | | Run | 12 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.681 | 10.7061 | 0.0253 | | 1 | 10.7114 | 10.7368 | 0.0254 |
| 2 | 10.654 | 10.6802 | 0.026 | | 2 | 10.7566 | 10.7811 | 0.0245 |
| 3 | 10.8 | 10.8251 | 0.0254 | | 3 | 10.7303 | 10.755 | 0.0247 |
| 4 | 10.804 | 10.8295 | 0.0252 | | 4 | 10.6992 | 10.7239 | 0.0247 |
| 5 | 10.655 | 10.6806 | 0.0252 | | 5 | 10.5784 | 10.6033 | 0.0249 |
| 6 | 10.717 | 10.7423 | 0.0253 | | 6 | 10.71 | 10.7347 | 0.0247 |
| 7 | 10.641 | 10.6661 | 0.0251 | | 7 | 10.6661 | 10.6908 | 0.0247 |
| 8 | 10.729 | 10.7541 | 0.0253 | | 8 | 10.7179 | 10.7428 | 0.0249 |
| 9 | 10.617 | 10.6468 | 0.0299 | | 9 | 10.7486 | 10.7734 | 0.0248 |
| 10 | 10.669 | 10.6981 | 0.0289 | | 10 | 10.6676 | 10.6922 | 0.0246 |

**Table 9.8 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [2/10]**

| Run | 13 | | | | Run | 14 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.733 | 10.7665 | 0.034 | | 1 | 10.6639 | 10.6922 | 0.0283 |
| 2 | 10.63 | 10.6934 | 0.0637 | | 2 | 10.6907 | 10.719 | 0.0283 |
| 3 | 10.667 | 10.7013 | 0.0341 | | 3 | 10.7089 | 10.7378 | 0.0289 |
| 4 | 10.782 | 10.8147 | 0.0326 | | 4 | 10.6804 | 10.7086 | 0.0282 |
| 5 | 10.803 | 10.835 | 0.0317 | | 5 | 10.6995 | 10.7277 | 0.0282 |
| 6 | 10.583 | 10.6149 | 0.0324 | | 6 | 10.6194 | 10.6477 | 0.0283 |
| 7 | 10.667 | 10.6984 | 0.0318 | | 7 | 10.5781 | 10.6069 | 0.0288 |
| 8 | 10.691 | 10.7244 | 0.0332 | | 8 | 10.5917 | 10.6196 | 0.0279 |
| 9 | 10.732 | 10.7645 | 0.0327 | | 9 | 10.6163 | 10.6447 | 0.0284 |
| 10 | 10.769 | 10.7943 | 0.0255 | | 10 | 10.5987 | 10.622 | 0.0233 |
| | | | | | | | | |
| Run | 15 | | | | Run | 16 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.784 | 10.8105 | 0.0269 | | 1 | 10.8586 | 10.8921 | 0.0335 |
| 2 | 10.711 | 10.7383 | 0.0276 | | 2 | 10.7302 | 10.7636 | 0.0334 |
| 3 | 10.657 | 10.685 | 0.028 | | 3 | 10.6176 | 10.6521 | 0.0345 |
| 4 | 10.673 | 10.6998 | 0.0266 | | 4 | 10.6305 | 10.6639 | 0.0334 |
| 5 | 10.703 | 10.73 | 0.027 | | 5 | 10.7782 | 10.8115 | 0.0333 |
| 6 | 10.706 | 10.7332 | 0.0273 | | 6 | 10.6206 | 10.6549 | 0.0343 |
| 7 | 10.639 | 10.6666 | 0.0274 | | 7 | 10.7852 | 10.8188 | 0.0336 |
| 8 | 10.626 | 10.6543 | 0.0279 | | 8 | 10.6595 | 10.693 | 0.0335 |
| 9 | 10.803 | 10.8299 | 0.0269 | | 9 | 10.6987 | 10.73 | 0.0313 |
| 10 | 10.716 | 10.7393 | 0.0233 | | 10 | 10.7556 | 10.7824 | 0.0268 |
| | | | | | | | | |
| Run | 17 | | | | Run | 18 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.717 | 10.7476 | 0.0303 | | 1 | 10.798 | | -10.8 |
| 2 | 10.579 | 10.6104 | 0.0313 | | 2 | 10.6759 | | -10.68 |
| 3 | 10.78 | 10.8098 | 0.0295 | | 3 | 10.6301 | | -10.63 |
| 4 | 10.59 | 10.6086 | 0.0182 | | 4 | 10.6211 | | -10.62 |
| 5 | 10.656 | 10.6844 | 0.0283 | | 5 | 10.7008 | 10.7325 | 0.0317 |
| 6 | 10.692 | 10.718 | 0.0262 | | 6 | 10.82 | 10.8517 | 0.0317 |
| 7 | 10.572 | 10.6022 | 0.0302 | | 7 | 10.6716 | | -10.67 |
| 8 | 10.641 | 10.6717 | 0.0304 | | 8 | 10.6755 | | -10.68 |
| 9 | 10.725 | 10.7521 | 0.0268 | | 9 | 10.6422 | 10.6741 | 0.0319 |
| 10 | 10.692 | 10.7194 | 0.0277 | | 10 | 10.5988 | | -10.6 |

**Table 9.9 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [3/10]**

| Run | 19 | | | | Run | 20 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.624 | 10.6493 | 0.0252 | | 1 | 10.6753 | 10.7006 | 0.0253 |
| 2 | 10.758 | 10.7842 | 0.0258 | | 2 | 10.825 | 10.8508 | 0.0258 |
| 3 | 10.772 | 10.7969 | 0.0254 | | 3 | 10.7264 | 10.7522 | 0.0258 |
| 4 | 10.664 | 10.6896 | 0.0256 | | 4 | 10.6968 | 10.7224 | 0.0256 |
| 5 | 10.661 | 10.6866 | 0.0258 | | 5 | 10.6456 | 10.6712 | 0.0256 |
| 6 | 10.623 | 10.6486 | 0.0256 | | 6 | 10.6538 | 10.6795 | 0.0257 |
| 7 | 10.646 | 10.6718 | 0.0257 | | 7 | 10.6814 | 10.7072 | 0.0258 |
| 8 | 10.775 | 10.8 | 0.0254 | | 8 | 10.6845 | 10.7085 | 0.024 |
| 9 | 10.656 | 10.6821 | 0.0257 | | 9 | 10.698 | 10.7236 | 0.0256 |
| 10 | 10.714 | 10.744 | 0.0302 | | 10 | 10.6235 | 10.6501 | 0.0266 |
| | | | | | | | | |
| Run | 21 | | | | Run | 22 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.629 | 10.6567 | 0.028 | | 1 | 10.7069 | 10.7349 | 0.028 |
| 2 | 10.589 | 10.6164 | 0.0277 | | 2 | 10.5706 | 10.5983 | 0.0277 |
| 3 | 10.559 | 10.5871 | 0.0277 | | 3 | 10.5922 | 10.62 | 0.0278 |
| 4 | 10.744 | 10.7718 | 0.0281 | | 4 | 10.6562 | 10.684 | 0.0278 |
| 5 | 10.586 | 10.6142 | 0.0278 | | 5 | 10.7093 | 10.7367 | 0.0274 |
| 6 | 10.665 | 10.6931 | 0.0281 | | 6 | 10.6309 | 10.6584 | 0.0275 |
| 7 | 10.488 | 10.5149 | 0.0274 | | 7 | 10.5977 | 10.6259 | 0.0282 |
| 8 | 10.647 | 10.675 | 0.0277 | | 8 | 10.6402 | 10.6676 | 0.0274 |
| 9 | 10.679 | 10.7071 | 0.0283 | | 9 | 10.6418 | 10.669 | 0.0272 |
| 10 | 10.664 | 10.6946 | 0.0311 | | 10 | 10.6863 | 10.7028 | 0.0165 |
| | | | | | | | | |
| Run | 23 | | | | Run | 24 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.666 | 10.6923 | 0.0262 | | 1 | 10.7026 | 10.7356 | 0.033 |
| 2 | 10.615 | 10.641 | 0.0264 | | 2 | 10.6457 | 10.6773 | 0.0316 |
| 3 | 10.696 | 10.7221 | 0.026 | | 3 | 10.5561 | 10.5897 | 0.0336 |
| 4 | 10.751 | 10.7771 | 0.0262 | | 4 | 10.6402 | 10.6731 | 0.0329 |
| 5 | 10.728 | 10.7532 | 0.0256 | | 5 | 10.6306 | 10.6619 | 0.0313 |
| 6 | 10.722 | 10.7473 | 0.0257 | | 6 | 10.7305 | 10.7635 | 0.033 |
| 7 | 10.666 | 10.6922 | 0.0259 | | 7 | 10.6078 | 10.6406 | 0.0328 |
| 8 | 10.635 | 10.6607 | 0.0259 | | 8 | 10.6396 | 10.6726 | 0.033 |
| 9 | 10.671 | 10.6975 | 0.0261 | | 9 | 10.6982 | 10.7313 | 0.0331 |
| 10 | 10.624 | 10.6484 | 0.0246 | | 10 | 10.5642 | 10.6001 | 0.0359 |

**Table 9.10 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [4/10]**

| Run | 25 | | | | Run | 26 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.651 | 10.676 | 0.0254 | | 1 | 10.7209 | 10.7534 | 0.0325 |
| 2 | 10.609 | 10.6336 | 0.0247 | | 2 | 10.7134 | 10.7467 | 0.0333 |
| 3 | 10.68 | 10.7049 | 0.0254 | | 3 | 10.6524 | 10.6858 | 0.0334 |
| 4 | 10.641 | 10.6652 | 0.0247 | | 4 | 10.7367 | 10.7684 | 0.0317 |
| 5 | 10.621 | 10.6466 | 0.0252 | | 5 | 10.7392 | 10.7718 | 0.0326 |
| 6 | 10.619 | 10.6435 | 0.0248 | | 6 | 10.6202 | 10.6526 | 0.0324 |
| 7 | 10.642 | 10.6674 | 0.0256 | | 7 | 10.6031 | 10.6367 | 0.0336 |
| 8 | 10.635 | 10.6598 | 0.0245 | | 8 | 10.7155 | 10.7693 | 0.0538 |
| 9 | 10.637 | 10.6632 | 0.0261 | | 9 | 10.692 | 10.728 | 0.036 |
| 10 | 10.724 | 10.7545 | 0.0304 | | 10 | 0.679 | 10.7125 | 10.034 |
| | | | | | | | | |
| Run | 27 | | | | Run | 28 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.638 | 10.669 | 0.0315 | | 1 | 10.6112 | 10.6353 | 0.0241 |
| 2 | 10.618 | 10.6463 | 0.0285 | | 2 | 10.7014 | 10.7285 | 0.0271 |
| 3 | 10.736 | 10.7674 | 0.0311 | | 3 | 10.6182 | 10.645 | 0.0268 |
| 4 | 10.64 | 10.6699 | 0.0304 | | 4 | 10.7551 | 10.78 | 0.0249 |
| 5 | 10.596 | 10.6271 | 0.031 | | 5 | 10.6356 | 10.6608 | 0.0252 |
| 6 | 10.665 | 10.6968 | 0.0318 | | 6 | 10.5549 | 10.5815 | 0.0266 |
| 7 | 10.775 | 10.8066 | 0.032 | | 7 | 10.713 | 10.7391 | 0.0261 |
| 8 | 10.664 | 10.6956 | 0.0314 | | 8 | 10.6965 | 10.7224 | 0.0259 |
| 9 | 10.706 | 10.7348 | 0.0285 | | 9 | 10.6557 | 10.682 | 0.0263 |
| 10 | 10.704 | 10.739 | 0.035 | | 10 | 10.7503 | 10.7847 | 0.0344 |
| | | | | | | | | |
| Run | 29 | | | | Run | 30 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.751 | 10.7889 | 0.0375 | | 1 | 10.6069 | 10.6346 | 0.0277 |
| 2 | 10.636 | 10.6693 | 0.0331 | | 2 | 10.6456 | 10.6737 | 0.0281 |
| 3 | 10.553 | 10.5868 | 0.0336 | | 3 | 10.6806 | 10.7093 | 0.0287 |
| 4 | 10.583 | 10.6171 | 0.0343 | | 4 | 10.492 | 10.5202 | 0.0282 |
| 5 | 10.706 | 10.7394 | 0.0334 | | 5 | 10.6422 | 10.6716 | 0.0294 |
| 6 | 10.66 | 10.6951 | 0.0355 | | 6 | 10.6518 | 10.6795 | 0.0277 |
| 7 | 10.636 | 10.6985 | 0.0628 | | 7 | 10.6586 | 10.6874 | 0.0288 |
| 8 | 10.65 | 10.6844 | 0.0344 | | 8 | 10.7197 | 10.7489 | 0.0292 |
| 9 | 10.679 | 10.7125 | 0.0333 | | 9 | 10.6926 | 10.7226 | 0.03 |
| 10 | 10.779 | 10.8118 | 0.0325 | | 10 | 10.6404 | 10.6737 | 0.0333 |

**Table 9.11 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [5/10]**

| Run | 31 | | | | Run | 32 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.657 | 10.6818 | 0.0244 | | 1 | 10.7451 | 10.7783 | 0.0332 |
| 2 | 10.609 | 10.6341 | 0.0253 | | 2 | 10.663 | 10.6967 | 0.0337 |
| 3 | 10.709 | 10.7334 | 0.0243 | | 3 | 10.6825 | 10.7162 | 0.0337 |
| 4 | 10.778 | 10.8039 | 0.0258 | | 4 | 10.7116 | 10.7448 | 0.0332 |
| 5 | 10.703 | 10.7284 | 0.0259 | | 5 | 10.7265 | 10.7602 | 0.0337 |
| 6 | 10.659 | 10.6848 | 0.0261 | | 6 | 10.5913 | 10.6247 | 0.0334 |
| 7 | 10.649 | 10.6738 | 0.0244 | | 7 | 10.5805 | 10.6135 | 0.033 |
| 8 | 10.593 | 10.6185 | 0.026 | | 8 | 10.721 | 10.7544 | 0.0334 |
| 9 | 10.689 | 10.7155 | 0.0261 | | 9 | 10.6207 | 10.6563 | 0.0356 |
| 10 | 10.636 | 10.6615 | 0.0258 | | 10 | 10.658 | 10.6863 | 0.0283 |
| | | | | | | | | |
| Run | 33 | | | | Run | 34 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.653 | 10.6805 | 0.0278 | | 1 | 10.615 | 10.6478 | 0.0328 |
| 2 | 10.585 | 10.6128 | 0.0276 | | 2 | 10.7222 | 10.7514 | 0.0292 |
| 3 | 10.536 | 10.5634 | 0.0273 | | 3 | 10.7883 | 10.8204 | 0.0321 |
| 4 | 10.638 | 10.6646 | 0.0268 | | 4 | 10.6146 | 10.647 | 0.0324 |
| 5 | 10.644 | 10.6727 | 0.0283 | | 5 | 10.6353 | 10.6682 | 0.0329 |
| 6 | 10.726 | 10.7531 | 0.0273 | | 6 | 10.6947 | 10.7277 | 0.033 |
| 7 | 10.653 | 10.681 | 0.0277 | | 7 | 10.5553 | 10.5882 | 0.0329 |
| 8 | 10.589 | 10.6158 | 0.027 | | 8 | 10.722 | 10.7545 | 0.0325 |
| 9 | 10.69 | 10.7165 | 0.027 | | 9 | 10.7198 | 10.7527 | 0.0329 |
| 10 | 10.572 | 10.6013 | 0.0291 | | 10 | 10.6253 | 10.6962 | 0.0709 |
| | | | | | | | | |
| Run | 35 | | | | Run | 36 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.692 | 10.7134 | 0.0219 | | 1 | 10.6614 | 10.69 | 0.0286 |
| 2 | 10.713 | 10.7364 | 0.0238 | | 2 | 10.6548 | 10.683 | 0.0282 |
| 3 | 10.649 | 10.6724 | 0.0239 | | 3 | 10.654 | 10.6826 | 0.0286 |
| 4 | 10.687 | 10.7107 | 0.0238 | | 4 | 10.7061 | 10.7347 | 0.0286 |
| 5 | 10.618 | 10.6413 | 0.0233 | | 5 | 10.6576 | 10.6857 | 0.0281 |
| 6 | 10.558 | 10.5816 | 0.0237 | | 6 | 10.7051 | 10.7061 | 0.001 |
| 7 | 10.692 | 10.716 | 0.0238 | | 7 | 10.6275 | 10.6558 | 0.0283 |
| 8 | 10.635 | 10.6579 | 0.0234 | | 8 | 10.7257 | 10.7547 | 0.029 |
| 9 | 10.647 | 10.6708 | 0.0236 | | 9 | 10.6369 | 10.6667 | 0.0298 |
| 10 | 10.747 | 10.7787 | 0.0315 | | 10 | 10.7175 | 10.7473 | 0.0298 |

**Table 9.12 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [6/10]**

| Run | 37 | | | | Run | 38 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.673 | 10.703 | 0.03 | | 1 | 10.7183 | 10.7488 | 0.0305 |
| 2 | 10.574 | 10.6018 | 0.028 | | 2 | 10.7005 | 10.7312 | 0.0307 |
| 3 | 10.611 | 10.6421 | 0.0308 | | 3 | 10.7353 | 10.764 | 0.0287 |
| 4 | 10.655 | 10.6844 | 0.0291 | | 4 | 10.6853 | 10.7158 | 0.0305 |
| 5 | 10.754 | 10.7845 | 0.0308 | | 5 | 10.5852 | 10.6152 | 0.03 |
| 6 | 10.81 | 10.8409 | 0.0308 | | 6 | 10.722 | 10.7521 | 0.0301 |
| 7 | 10.637 | 10.6666 | 0.0298 | | 7 | 10.712 | 10.7426 | 0.0306 |
| 8 | 10.6 | 10.6289 | 0.0288 | | 8 | 10.7169 | 10.7449 | 0.028 |
| 9 | 10.656 | 10.683 | 0.0271 | | 9 | 10.8203 | 10.8508 | 0.0305 |
| 10 | 10.748 | 10.7809 | 0.0325 | | 10 | 10.6847 | 10.7153 | 0.0306 |
| | | | | | | | | |
| Run | 39 | | | | Run | 40 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.576 | 10.6027 | 0.0263 | | 1 | 10.6372 | 10.6623 | 0.0251 |
| 2 | 10.666 | 10.6931 | 0.0267 | | 2 | | 10.6657 | 10.666 |
| 3 | 10.615 | 10.6413 | 0.0265 | | 3 | | 10.6764 | 10.676 |
| 4 | 10.708 | 10.7345 | 0.0264 | | 4 | 10.67 | 10.6907 | 0.0207 |
| 5 | 10.666 | 10.692 | 0.0264 | | 5 | | 10.594 | 10.594 |
| 6 | 10.573 | 10.5995 | 0.0262 | | 6 | | 10.6571 | 10.657 |
| 7 | 10.64 | 10.6662 | 0.0265 | | 7 | 10.624 | 10.6379 | 0.0139 |
| 8 | 10.673 | 10.7 | 0.0266 | | 8 | | 10.7031 | 10.703 |
| 9 | 10.699 | 10.725 | 0.0262 | | 9 | | 10.765 | 10.765 |
| 10 | 10.728 | 10.7586 | 0.0305 | | 10 | | 10.6585 | 10.659 |
| | | | | | | | | |
| Run | 41 | | | | Run | 42 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.689 | 10.7194 | 0.0308 | | 1 | 10.7119 | 10.7405 | 0.0286 |
| 2 | 10.695 | 10.7256 | 0.0311 | | 2 | 10.7578 | 10.7861 | 0.0283 |
| 3 | 10.707 | 10.7376 | 0.0308 | | 3 | 10.7136 | 10.7419 | 0.0283 |
| 4 | 10.696 | 10.7257 | 0.0296 | | 4 | 10.8086 | 10.837 | 0.0284 |
| 5 | 10.757 | 10.7862 | 0.0297 | | 5 | 10.6579 | 10.6862 | 0.0283 |
| 6 | 10.632 | 10.6616 | 0.0298 | | 6 | 10.6323 | 10.6608 | 0.0285 |
| 7 | 10.63 | 10.6608 | 0.031 | | 7 | 10.698 | 10.7264 | 0.0284 |
| 8 | 10.717 | 10.7476 | 0.0305 | | 8 | 10.5545 | 10.5834 | 0.0289 |
| 9 | 10.669 | 10.699 | 0.0305 | | 9 | 10.6076 | 10.6365 | 0.0289 |
| 10 | 10.578 | 10.7104 | 0.1324 | | 10 | 10.7722 | 10.803 | 0.0308 |

**Table 9.13 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [7/10]**

| Run | 43 | | | | Run | 44 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.683 | 10.7124 | 0.0298 | | 1 | 10.7286 | 10.7532 | 0.0246 |
| 2 | 10.732 | 10.7626 | 0.0307 | | 2 | 10.5226 | 10.5469 | 0.0243 |
| 3 | 10.622 | 10.652 | 0.0303 | | 3 | 10.7207 | 10.745 | 0.0243 |
| 4 | 10.79 | 10.82 | 0.0298 | | 4 | 10.6838 | 10.7083 | 0.0245 |
| 5 | 10.703 | 10.7302 | 0.0277 | | 5 | 10.6725 | 10.6968 | 0.0243 |
| 6 | 10.774 | 10.8047 | 0.0303 | | 6 | 10.7257 | 10.7502 | 0.0245 |
| 7 | 10.736 | 10.7658 | 0.0303 | | 7 | 10.6757 | 10.7001 | 0.0244 |
| 8 | 10.564 | 10.5942 | 0.0301 | | 8 | 10.6829 | 10.7073 | 0.0244 |
| 9 | 10.584 | 10.6144 | 0.0306 | | 9 | 10.693 | 10.7174 | 0.0244 |
| 10 | 10.743 | 10.7698 | 0.0266 | | 10 | 10.7245 | 10.751 | 0.0265 |
| | | | | | | | | |
| Run | 45 | | | | Run | 46 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.704 | 10.7312 | 0.0269 | | 1 | 10.7513 | 10.7815 | 0.0302 |
| 2 | 10.766 | 10.7907 | 0.0247 | | 2 | 10.6459 | 10.6746 | 0.0287 |
| 3 | 10.663 | 10.6869 | 0.0242 | | 3 | 10.5809 | 10.6114 | 0.0305 |
| 4 | 10.535 | 10.5608 | 0.0256 | | 4 | 10.5407 | 10.5704 | 0.0297 |
| 5 | 10.543 | 10.57 | 0.0274 | | 5 | 10.6463 | 10.6763 | 0.03 |
| 6 | 10.609 | 10.636 | 0.027 | | 6 | 10.6753 | 10.709 | 0.0337 |
| 7 | 10.656 | 10.6804 | 0.0246 | | 7 | 10.5662 | 10.5964 | 0.0302 |
| 8 | 10.573 | 10.5991 | 0.0265 | | 8 | 10.6488 | 10.6788 | 0.03 |
| 9 | 10.615 | 10.6394 | 0.0247 | | 9 | 10.6046 | 10.6347 | 0.0301 |
| 10 | 10.598 | 10.6177 | 0.02 | | 10 | 10.5737 | 10.6034 | 0.0297 |
| | | | | | | | | |
| | | | | | | | | |
| Run | 47 | | | | Run | 48 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.57 | 10.596 | 0.026 | | 1 | 10.6746 | 10.7016 | 0.027 |
| 2 | 10.717 | 10.7549 | 0.0382 | | 2 | 10.5486 | 10.5748 | 0.0262 |
| 3 | 10.717 | 10.7572 | 0.0404 | | 3 | 10.6567 | 10.6829 | 0.0262 |
| 4 | 10.643 | 10.7114 | 0.0688 | | 4 | 10.6718 | 10.698 | 0.0262 |
| 5 | 10.656 | 10.8485 | 0.1927 | | 5 | 10.6774 | 10.7036 | 0.0262 |
| 6 | 10.604 | 10.6727 | 0.0686 | | 6 | 10.5758 | 10.6025 | 0.0267 |
| 7 | 10.694 | 10.7227 | 0.029 | | 7 | 10.6876 | 10.7137 | 0.0261 |
| 8 | 10.594 | 10.6298 | 0.0361 | | 8 | 10.7212 | 10.7474 | 0.0262 |
| 9 | 10.6 | 10.6748 | 0.0744 | | 9 | 10.7532 | 10.7795 | 0.0263 |
| 10 | 10.712 | 10.7389 | 0.027 | | 10 | 10.6901 | 10.7206 | 0.0305 |

**Table 9.14 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [8/10]**

| Run | 49 | | | | Run | 50 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.633 | 10.6588 | 0.026 | | 1 | 10.7405 | 10.7635 | 0.023 |
| 2 | 10.642 | 10.6681 | 0.0265 | | 2 | 10.6557 | 10.6779 | 0.0222 |
| 3 | 10.664 | 10.6894 | 0.025 | | 3 | 10.6834 | 10.7058 | 0.0224 |
| 4 | 10.668 | 10.6939 | 0.026 | | 4 | 10.6685 | 10.6903 | 0.0218 |
| 5 | 10.672 | 10.6984 | 0.026 | | 5 | 10.4887 | 10.511 | 0.0223 |
| 6 | 10.682 | 10.7075 | 0.026 | | 6 | 10.647 | 10.6685 | 0.0215 |
| 7 | 10.715 | 10.7402 | 0.0253 | | 7 | 10.6642 | 10.6861 | 0.0219 |
| 8 | 10.697 | 10.7228 | 0.026 | | 8 | 10.6803 | 10.702 | 0.0217 |
| 9 | 10.684 | 10.7096 | 0.0258 | | 9 | 10.5842 | 10.608 | 0.0238 |
| 10 | 10.618 | 10.648 | 0.0296 | | 10 | 10.6611 | 10.6923 | 0.0312 |
| | | | | | | | | |
| Run | 51 | | | | Run | 52 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.704 | 10.7298 | 0.0258 | | 1 | 10.7202 | 10.7492 | 0.029 |
| 2 | 10.719 | 10.7449 | 0.026 | | 2 | 10.6461 | 10.6746 | 0.0285 |
| 3 | 10.622 | 10.6486 | 0.0263 | | 3 | 10.546 | 10.5745 | 0.0285 |
| 4 | 10.542 | 10.5677 | 0.0257 | | 4 | 10.614 | 10.6428 | 0.0288 |
| 5 | 10.716 | 10.7415 | 0.026 | | 5 | 10.7502 | 10.7793 | 0.0291 |
| 6 | 10.67 | 10.696 | 0.0261 | | 6 | 10.5985 | 10.6275 | 0.029 |
| 7 | 10.701 | 10.7262 | 0.0252 | | 7 | 10.5809 | 10.609 | 0.0281 |
| 8 | 10.621 | 10.647 | 0.0262 | | 8 | 10.6337 | 10.6624 | 0.0287 |
| 9 | 10.559 | 10.5846 | 0.0258 | | 9 | 10.6356 | 10.6642 | 0.0286 |
| 10 | 10.639 | 10.6659 | 0.0271 | | 10 | 10.6087 | 10.6387 | 0.03 |
| | | | | | | | | |
| Run | 53 | | | | Run | 54 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.692 | 10.7201 | 0.0277 | | 1 | 10.6873 | | -10.69 |
| 2 | 10.703 | 10.7304 | 0.0277 | | 2 | 10.6861 | 10.7172 | 0.0311 |
| 3 | 10.66 | 10.6878 | 0.0283 | | 3 | 10.6979 | | -10.7 |
| 4 | 10.689 | 10.7168 | 0.0276 | | 4 | 10.6392 | | -10.64 |
| 5 | 10.675 | 10.7022 | 0.0276 | | 5 | 10.605 | 10.6377 | 0.0327 |
| 6 | 10.602 | 10.6277 | 0.0255 | | 6 | 10.6836 | 10.7162 | 0.0326 |
| 7 | 10.638 | 10.6662 | 0.0286 | | 7 | 10.7549 | | -10.75 |
| 8 | 10.583 | 10.6111 | 0.0281 | | 8 | 10.6926 | | -10.69 |
| 9 | 10.521 | 10.5488 | 0.0274 | | 9 | 10.6875 | | -10.69 |
| 10 | 10.599 | 10.6257 | 0.0263 | | 10 | 10.5643 | | -10.56 |

**Table 9.15 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [9/10]**

| Run | 55 | | | | Run | 56 | | |
|---|---|---|---|---|---|---|---|---|
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.674 | | -10.674 | | 1 | 10.7187 | 10.7492 | 0.0305 |
| 2 | 10.776 | | -10.776 | | 2 | 10.639 | 10.6703 | 0.0313 |
| 3 | 10.688 | | -10.688 | | 3 | 10.6098 | 10.6415 | 0.0317 |
| 4 | 10.637 | | -10.637 | | 4 | 10.6522 | 10.6836 | 0.0314 |
| 5 | 10.688 | | -10.688 | | 5 | 10.6042 | 10.635 | 0.0308 |
| 6 | 10.546 | 10.5789 | 0.0328 | | 6 | 10.6648 | 10.6924 | 0.0276 |
| 7 | 10.628 | 10.6605 | 0.0328 | | 7 | 10.5981 | 10.6291 | 0.031 |
| 8 | 10.704 | 10.7365 | 0.033 | | 8 | 10.518 | 10.5479 | 0.0299 |
| 9 | 10.622 | | -10.622 | | 9 | 10.632 | 10.6626 | 0.0306 |
| 10 | 10.572 | | -10.572 | | 10 | 10.6507 | 10.6724 | 0.0217 |
| | | | | | | | | |
| | | | | | | | | |
| Run | 57 | | | | Run | 58 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.669 | 10.6926 | 0.0241 | | 1 | 10.6556 | | -10.66 |
| 2 | 10.574 | 10.6011 | 0.0267 | | 2 | 10.6747 | 10.7058 | 0.0311 |
| 3 | 10.692 | 10.7163 | 0.0239 | | 3 | 10.6187 | 10.6498 | 0.0311 |
| 4 | 10.636 | 10.6627 | 0.0265 | | 4 | 10.6943 | 10.7259 | 0.0316 |
| 5 | 10.684 | 10.71 | 0.0265 | | 5 | 10.662 | | -10.66 |
| 6 | 10.698 | 10.7244 | 0.0268 | | 6 | 10.6769 | | -10.68 |
| 7 | 10.616 | 10.6426 | 0.0269 | | 7 | 10.6485 | | -10.65 |
| 8 | 10.756 | 10.7806 | 0.0247 | | 8 | 10.8188 | | -10.82 |
| 9 | 10.795 | 10.8192 | 0.024 | | 9 | 10.7162 | | -10.72 |
| 10 | 10.632 | 10.6539 | 0.0216 | | 10 | 10.6581 | | -10.66 |
| | | | | | | | | |
| Run | 59 | | | | Run | 60 | | |
| Vial | Mass Empty | Mass post fd | Mass Solid | | Vial | Mass Empty | Mass post fd | Mass Solid |
| 1 | 10.703 | 10.7266 | 0.024 | | 1 | 10.6701 | | -10.67 |
| 2 | 10.651 | 10.6746 | 0.0232 | | 2 | 10.5963 | | -10.6 |
| 3 | 10.641 | 10.6641 | 0.0235 | | 3 | 10.7228 | 10.757 | 0.0342 |
| 4 | 10.626 | 10.6501 | 0.0244 | | 4 | 10.7029 | | -10.7 |
| 5 | 10.785 | 10.8022 | 0.0172 | | 5 | 10.6597 | 10.6936 | 0.0339 |
| 6 | 10.679 | 10.7019 | 0.0233 | | 6 | 10.7061 | 10.7403 | 0.0342 |
| 7 | 10.714 | 10.7375 | 0.0235 | | 7 | 10.6752 | | -10.68 |
| 8 | 10.674 | 10.6977 | 0.0234 | | 8 | 10.584 | | -10.58 |
| 9 | 10.62 | 10.6564 | 0.0364 | | 9 | 10.6257 | | -10.63 |
| 10 | 10.63 | 10.6564 | 0.0264 | | 10 | 10.6322 | | -10.63 |

**Table 9.16 Mass of solid in vials used in Chapter 5 for Karl Fischer Titration. The run numbers refer to the cycles shown in Table 5.2 [10/10]**

| Vial | Mass | % Moisture | Average | Vial | Mass | % Moisture | Average | Vial | Mass | % Moisture | Average | Vial |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.4 | 0.0415 | 27.95 | 23.04 | 11.3 | 0.0254 | 4.34 | 4.77 | 21.1 | 0.028 | 3.88 | 3.90 | 31.7 |
| 1.5 | 0.0413 | 15.08 | | 11.5 | 0.0252 | 4.86 | | 21.2 | 0.0277 | 4.05 | | 32.2 |
| 1.3 | 0.0416 | 26.10 | | 11.8 | 0.0253 | 5.12 | | 21.9 | 0.0283 | 3.76 | | 32.3 |
| 2.1 | 0.0312 | 3.07 | 3.23 | 12.2 | 0.0245 | 2.94 | 3.89 | 22.2 | 0.0277 | 5.25 | 5.35 | 32.4 |
| 2.2 | 0.0308 | 3.32 | | 12.4 | 0.0247 | 4.03 | | 22.6 | 0.0275 | 4.37 | | 33.4 |
| 2.3 | 0.031 | 3.31 | | 12.9 | 0.0248 | 4.71 | | 22.7 | 0.0282 | 6.42 | | 33.5 |
| 3.5 | 0.031 | 20.22 | 21.59 | 13.5 | 0.0317 | 2.57 | 3.72 | 23.2 | 0.0264 | 3.64 | 3.67 | 33.6 |
| 3.6 | 0.0302 | 20.86 | | 13.7 | 0.0318 | 3.22 | | 23.6 | 0.0257 | 3.76 | | 34.2 |
| 3.9 | 0.0299 | 23.70 | | 13.9 | 0.0327 | 5.38 | | 23.9 | 0.0261 | 3.60 | | 34.5 |
| 4.1 | 0.0254 | 2.16 | 3.19 | 14.2 | 0.0283 | 9.72 | 10.31 | 24.2 | 0.0316 | 17.52 | 17.91 | 34.8 |
| 4.2 | 0.0163 | 3.88 | | 14.4 | 0.0282 | 10.17 | | 24.7 | 0.0328 | 19.17 | | 35.1 |
| 4.3 | 0.0265 | 3.52 | | 14.7 | 0.0288 | 11.04 | | 24.8 | 0.033 | 17.03 | | 35.3 |
| 5.1 | 0.0348 | 10.62 | 10.70 | 15.1 | 0.0269 | 6.94 | 8.36 | 25.1 | 0.0254 | 10.22 | 10.43 | 35.7 |
| 5.3 | 0.0352 | 11.25 | | 15.4 | 0.0266 | 7.51 | | 25.3 | 0.0254 | 10.27 | | 36.3 |
| 5.8 | 0.0348 | 10.23 | | 15.5 | 0.027 | 10.62 | | 25.7 | 0.0256 | 10.81 | | 36.4 |
| 6.3 | 0.0251 | 1.68 | 1.72 | 16.2 | 0.0334 | 5.66 | 6.36 | 26.2 | 0.0333 | 22.36 | 23.19 | 36.7 |
| 6.5 | 0.0254 | 1.98 | | 16.3 | 0.0345 | 6.42 | | 26.5 | 0.0326 | 24.44 | | 37.3 |
| 6.9 | 0.0259 | 1.50 | | 16.8 | 0.0335 | 7.01 | | 26.6 | 0.0324 | 22.78 | | 38.4 |
| 7.1 | 0.0274 | 16.39 | 13.97 | 17.3 | 0.0295 | 18.24 | 16.19 | 27.3 | 0.0311 | 30.80 | 30.80 | 39.1 |
| 7.4 | 0.0273 | 14.08 | | 17.8 | 0.0304 | 22.76 | | 28.3 | 0.0268 | 8.84 | 9.05 | 39.3 |
| 7.9 | 0.0272 | 11.45 | | 17.9 | 0.0268 | 7.58 | | 28.8 | 0.0259 | 9.44 | | 39.6 |
| 8.3 | 0.0329 | 9.56 | 5.48 | 18.5 | 0.0317 | 3.86 | 3.81 | 28.9 | 0.0263 | 8.88 | | 40.1 |
| 8.8 | 0.031 | 4.57 | | 18.6 | 0.0317 | 3.36 | | 29.1 | 0.0375 | 34.38 | 34.38 | 40.4 |
| 8.9 | 0.0303 | 2.30 | | 18.9 | 0.0319 | 4.22 | | 29.8 | 0.0344 | | | 40.7 |
| 9.3 | 0.0261 | 4.14 | 4.23 | 19.5 | 0.0258 | 7.40 | 7.21 | 29.9 | 0.0333 | | | 41.3 |
| 9.6 | 0.0263 | 4.30 | | 19.7 | 0.0257 | 7.14 | | 30.4 | 0.0282 | 8.81 | 10.38 | 41.4 |
| 9.8 | 0.0262 | 4.25 | | 19.8 | 0.0254 | 7.11 | | 30.6 | 0.0277 | 8.87 | | 41.8 |
| 10.3 | 0.0305 | 18.65 | 17.86 | 20.3 | 0.0258 | 15.41 | 14.76 | 30.8 | 0.0292 | 13.46 | | 42.3 |
| 10.5 | 0.0306 | 17.04 | | 20.5 | 0.0256 | 13.69 | | 31.2 | 0.0253 | 12.25 | 9.68 | 42.6 |
| 10.6 | 0.0298 | 17.89 | | 20.7 | 0.0258 | 15.17 | | 31.3 | 0.0243 | 7.93 | | 42.7 |

**Table 9.17 Raw Karl Fischer data for runs detailed in Table 5.2 [1/2]**

| Mass | % Moisture | Average | Vial | Mass | % Moisture | Average | Vial | Mass | % Moisture | Average |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.0244 | 8.86 | | 43.1 | 0.0298 | 15.44 | 17.95 | 52.5 | 0.0291 | 21.81 | |
| 0.0337 | 12.48 | 11.80 | 43.3 | 0.0303 | 20.46 | | 53.1 | 0.0277 | 13.61 | 11.48 |
| 0.0337 | 11.37 | | 43.8 | 0.0301 | 17.94 | | 53.2 | 0.0277 | 14.43 | |
| 0.0332 | 11.56 | | 44.3 | 0.0243 | 1.98 | 1.81 | 53.6 | 0.0255 | 6.39 | |
| 0.0268 | 12.74 | 12.47 | 44.5 | 0.0243 | 1.94 | | 54.2 | 0.0311 | 6.75 | 8.08 |
| 0.0283 | 11.48 | | 44.7 | 0.0244 | 1.51 | | 54.5 | 0.0327 | 8.29 | |
| 0.0273 | 13.18 | | 45.1 | 0.0269 | 17.43 | 11.06 | 54.6 | 0.0326 | 9.20 | |
| 0.0292 | 3.04 | 19.90 | 45.2 | 0.0247 | 6.05 | | 55.6 | 0.0328 | 5.75 | 5.69 |
| 0.0329 | 20.02 | | 45.3 | 0.0242 | 3.43 | | 55.7 | 0.0328 | 5.48 | |
| 0.0325 | 19.77 | | 45.5 | 0.0274 | 17.32 | | 55.8 | 0.033 | 5.85 | |
| 0.0219 | 12.53 | 17.40 | 46.3 | 0.0305 | 20.17 | 18.79 | 56.1 | 0.0305 | 11.99 | 11.44 |
| 0.0239 | 20.00 | | 46.8 | 0.03 | 18.20 | | 56.5 | 0.0308 | 12.35 | |
| 0.0238 | 19.67 | | 46.9 | 0.0301 | 18.00 | | 56.8 | 0.0299 | 9.99 | |
| 0.0286 | 11.59 | 11.10 | 47.1 | 0.026 | 19.70 | 22.93 | 57.4 | 0.0265 | 12.63 | 7.54 |
| 0.0286 | 11.48 | | 47.2 | 0.0382 | 31.75 | | 57.8 | 0.0247 | 6.77 | |
| 0.0283 | 10.23 | | 47.7 | 0.029 | 17.33 | | 57.9 | 0.024 | 3.22 | |
| 0.0308 | 20.16 | 20.16 | 48.3 | 0.0262 | 15.15 | 14.23 | 58.2 | 0.0311 | 4.96 | 5.20 |
| 0.0305 | 20.44 | 20.44 | 48.6 | 0.0267 | 14.62 | | 58.3 | 0.0311 | 5.60 | |
| 0.0263 | 8.37 | 9.53 | 48.7 | 0.0261 | 12.91 | | 58.4 | 0.0316 | 5.04 | |
| 0.0265 | 10.69 | | 49.3 | 0.025 | 5.92 | 8.39 | 59.3 | 0.0235 | 1.85 | 1.87 |
| 0.0262 | 9.53 | | 49.4 | 0.026 | 8.82 | | 59.7 | 0.0235 | 1.85 | |
| 0.0251 | 7.01 | 9.16 | 49.9 | 0.0258 | 10.43 | | 59.8 | 0.0234 | 1.90 | |
| 0.0207 | 9.42 | | 50.2 | 0.0222 | 21.68 | 20.42 | 60.3 | 0.0342 | 13.64 | 12.41 |
| 0.0139 | 11.04 | | 50.3 | 0.0224 | 20.02 | | 60.5 | 0.0339 | 11.10 | |
| 0.0308 | 21.08 | 19.86 | 50.6 | 0.0215 | 19.56 | | 60.6 | 0.0342 | 12.49 | |
| 0.0296 | 19.34 | | 51.1 | 0.0258 | 9.16 | 9.95 | | | | |
| 0.0305 | 19.17 | | 51.8 | 0.0262 | 13.59 | | | | | |
| 0.0283 | 12.29 | 12.44 | 51.9 | 0.0258 | 7.10 | | | | | |
| 0.0285 | 12.95 | | 52.3 | 0.0285 | 26.19 | 23.23 | | | | |
| 0.0284 | 12.09 | | 52.4 | 0.0288 | 21.68 | | | | | |

**Table 9.18 Raw Karl Fischer data for runs detailed in Table 5.2 [2/2]**

## Chapter 10. References

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